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THE SEATO MEDICAL RESEARCH LABORATORY

AND

THE SEATO CLINICAL RESEARCH CENTER

RAJVITHI ROAD
BANGKOK, THAILAND

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FORWORD

Men, women, and children are the critical resource of any nation. In a developing nation such as Thailand, human resources are the mainspring of social improvement, economic progress, and political survival. It is the initiative and know-how of the people that change a low-income country into a modern nation; but, education and opportunity are needlessly wasted if disease saps people of the ability and the desire to strive, both for the nation and themselves. A man with malaria may find that only a few hours of fishing or cultivating are possible each day. A child with chronic dysentery is a listless student.

It is the hope of men of good will everywhere that more and more the energies of all nations may be channeled to the constructive purpose of furthering the well-being of man. In this effort, increased teamwork is essential. This includes the teamwork of workers in the life sciences not only within a government, but also between the governments of the nations of the world themselves.

This report describes in general the team work of life sciences workers of the governments represented in the South-East Asia Treaty Organization, and specifically the activities of Thai and United States workers during the period 1 April 1966-31 March 1967.

JESUS VARGAS
Secretary General
SEATO

Disease and nutrition have influenced military campaigns throughout the history of the world. As late as World War II, battle decisions were influenced by diseases such as scrub typhus and dengue in the military troops. Certain areas of land mass were denied to military maneuver because of the over-whelming incidence of malaria. Today, malaria and other infectious diseases are better understood but are still diseases of major military medical importance. The soldier's abilities in combat is dependent upon his physical strength and endurance brought to a peak by training. The best training program and the most modern weapons will fail to achieve their objective if the soldier is not physically and mentally capable of benefiting from them.

P. Phintuyothin

PUNG PHINTUYOTHIN
Major General, MC, RTA
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Many diseases in Southeast Asia have significant socioeconomic effects. Some disease processes such as chronic infections and malnutrition are particularly debilitating, and may exert their influence throughout an individual's entire lifespan. Anemia is widespread, and may be due to a variety of causes including genetic abnormalities as well as infectious and nutritional deficiencies. If severe and early in onset, such processes can retard the physical development of the individual. Research leading to the improvement of health problems of this sort, and upon the reaction of man to disease in a tropical environment is provided for under the SEATO Medical Research Program. Through this research, in combination with medical education and training, an enduring contribution will be made to the health of man in Thailand and throughout all other SEATO nations.

Swasdi Skulthai

SWASDI SKULTHAI, MD.
Director-General
SEATO Clinical Research Center

THE SOUTH-EAST ASIA TREATY ORGANIZATION MEDICAL RESEARCH PROGRAM IN THAILAND

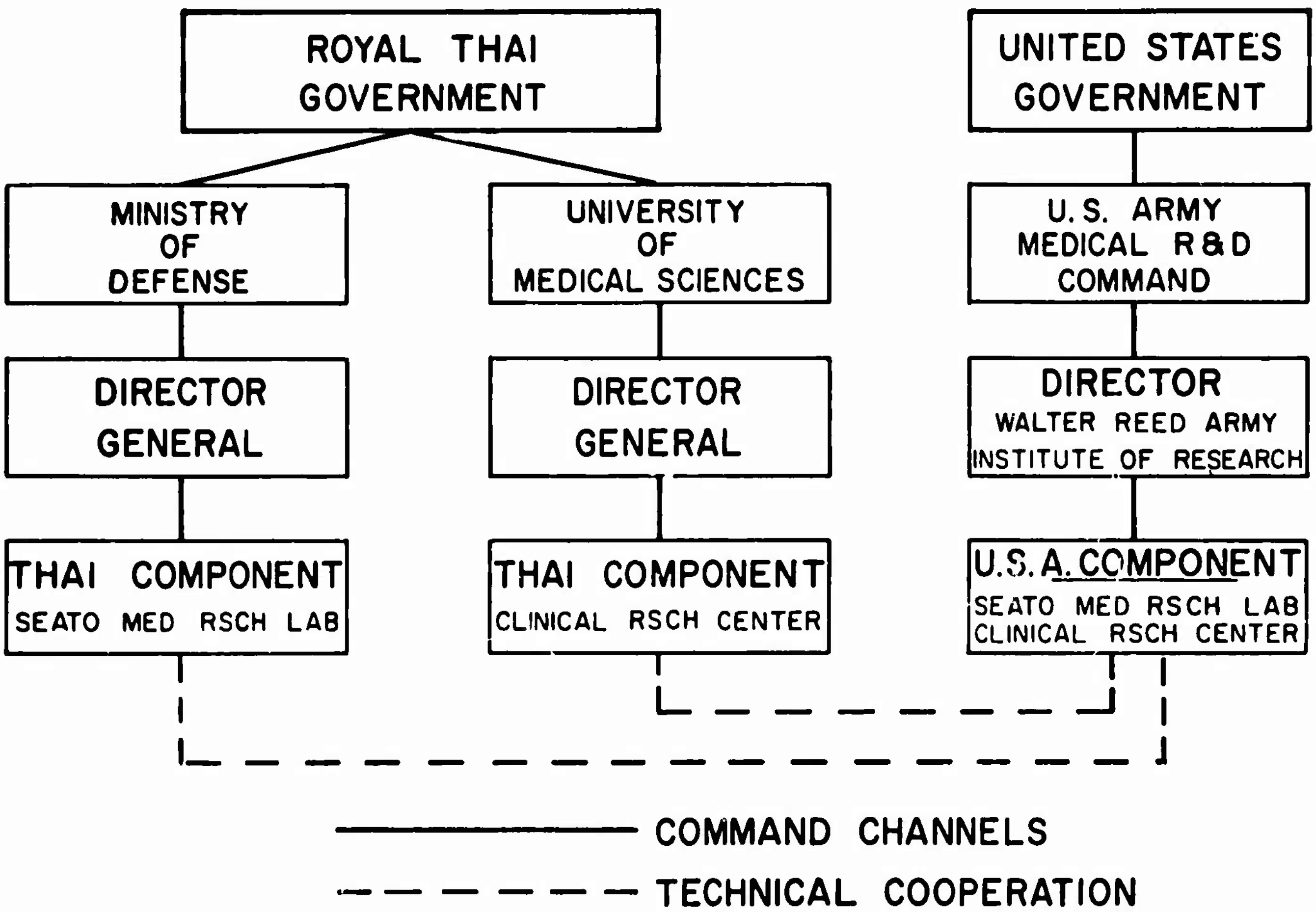

TABLE OF CONTENTS

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SEATO MEDICAL RESEARCH STUDY ON ARBOVIRUSES

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Period of Report: 1 April 1966 – 31 March 1967

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General Information

During the period covered by this report, studies on the epidemiology of dengue hemorrhagic fever in Thailand were continued in cooperation with the Departments of Entomology and Epidemiology of US Army Medical Component SEATO. An insular outbreak of hemorrhagic fever was studied in detail during the summer of 1966. In addition, through the assistance of the Public Health Division USAID and the Pasteur Institute, Vietnam, epidemiologic data on hemorrhagic fever in Saigon were obtained for comparison with similar data from Bangkok.

Laboratory studies on dengue virology included extensive investigation of a plaque reduction neutralization test and its applications for identification of dengue viruses and measurement of serum antibody. Investigation of cell culture methods for isolation and propagation of dengue viruses resulted in development of new and more sensitive techniques. Dengue infection in primates was studied for the purpose of determining the suitability of different species for cross-protection studies with dengue viruses and for production of reference antiserum.

A major effort has been made to develop the capability in this laboratory of studying in detail the immunoglobulin response in dengue fever and dengue hemorrhagic fever. Significant progress has been made in adaptation of density gradient centrifugation, gel filtration, ion-exchange chromatography, and immunoelectrophoresis methods to fit our requirements.

Studies of arbovirus diseases in Americans were concerned primarily with studies of fevers of unknown origin in US military personnel in the Republic of Vietnam. These studies were carried out in cooperation with personnel of the US Army Medical Research Team, Vietnam, and the Departments of Medicine at several evacuation hospitals and field hospitals in Vietnam. In addition to arbovirus diagnostic tests, the hemolytic test for leptospirosis was adapted to microtiter methods and routinely used. Serologic tests for scrub typhus were performed by the Thai Component of SMRL.

The preliminary phase of ecologic studies on Japanese encephalitis at Bang Phra, being carried out in cooperation with the Department of Entomology and the personnel of the Pasteur Institute, is nearing completion. The first year's program including mosquito collections, virus isolation attempts from mosquitoes, collection of serum from a large variety of vertebrates for virus isolation and serologic testing is completed. The laboratory phase of this program, identification of viral isolates and serologic testing of vertebrate sera, is as yet incomplete. The second phase of the field program which will be directed at a few highly suspect vertebrate species and their relationship to arthropod vectors is scheduled to begin in May or June 1967. Designs of these specific studies will depend on analysis of the as yet incomplete laboratory results,

Title: Epidemiology of Dengue Hemorrhagic Fever

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Objective:

To determine the factors which influence the occurrence and spread of mosquito-borne hemorrhagic fever in Southeast Asia.

Description:

Data on hospitalized hemorrhagic fever cases in Bangkok-Thonburi hospitals were collected by monthly visits to each hospital by public health nurses in the same manner as had been used since 1962. Reports on hemorrhagic fever cases occurring outside of Bangkok were requested from hospitals. Additional data were available from Department of Public Health records of reports from provincial health officers.

Reports of large or unusual outbreaks were investigated by a team consisting of at least one physician and two public health nurses. Attempts were made to collect clinical and epidemiologic information and obtain specimens for virologic studies.

Epidemiologic data on hemorrhagic fever in Saigon are being collected through the cooperation of the US Army Medical Research Team, Vietnam, and the Public Health Division of United States Agency for International Development, Saigon. USAID public health nurses collected data on hospitalized hemorrhagic fever cases by visiting hospitals weekly and reviewing clinical records.

An epidemic of hemorrhagic fever on the island of Koh Samui in the gulf of Thailand off the coast of Surat Thani province was investigated in detail by a team made up of personnel from the Departments of Epidemiology, Entomology and Virology. The purpose of the studies was to determine the epidemiologic patterns of dengue hemorrhagic fever in a relatively isolated population in which the disease had recently appeared, and to determine whether serologic response to infection and the epidemiologic patterns are consistent with the "second infection" hypothesis of the dengue shock syndrome. In addition, studies were carried out to determine the relative prevalence of *Aedes aegypti* and *Aedes albopictus* on the island and to obtain information concerning the role of *Aedes albopictus* as a dengue vector.

Progress:

Hemorrhagic Fever in Bangkok. During 1966 hemorrhagic fever continued as a major cause of morbidity and a significant cause of mortality in Thai Children. A total of 3,663 cases were admitted to hospitals in Bangkok and Thonburi with 139 fatalities. The case fatality rate in 1966 was 3.8% essentially the same as the rate of 3.9% seen in 1965.

The distribution of cases by month (Figure 1) shows the peak incidence in June through August which was simillar to the seasonal pattern seen from 1962 to 1964. There were a relatively large number of cases during the dry months from December 1965 to April 1966 and a sharp decrease in the dry season beginning November 1966.

It has become apparent from discussions with pediatricians at Children's Hospital and Chulalongkorn Hospital that admitting policies have been chunged since 1964. At these two hospitals, which care for approximately half of all hospitalized cases, prior to 1965 most cases diagnosed as hemorrhagic fever were admitted. During 1965 and 1966 most mild cases were treated as outpatients and only patients with shock, bleeding, or signs of impending severe illness were admitted. Therefore the gross figures for 1965 and 1966 are not directly comparable to previous years and tend to underestimate in 1965 and 1966 since they are based on a more severely ill group of patients.

Age distribution remains essential unchanged from previous years with over 50% of cases occurring in the 3-6 year age group. Age specific rates given in Table 1 are based on the 1960 census.

Hemorrhagic Fever in Udorn Thani. In June-July 1966 hemorrhagic fever was reported for the first time in and near the city of Udorn Thani in Northeast Thailand. Clinical descriptions of cases were typical of hemorrhagic fever and serologic studies on 9 cases confirmed the dengue etiology.

Hemorrhagic Fever in Saigon. During 1966, 457 cases of hemorrhagic fever were admitted to 3 Saigon hospitals (Nhi Doug, Grall, and seventh Day Adventist). Of the 457 cases, 214 had clinical evidence of shock and there were 67 deaths reported. Distribution of cases by month, shown in Figure 2, reveals the majority of cases occurred during the southwest monsoon season (May to September) with a sharp peak in June and July. The seasonal variation is very simillar to that seen in Bangkok.

Age distribution (Table 2) is also very simillar to that seen in Bangkok except the relative number of cases under one year is lower.

The mortality rate in Saigon of 14.9% is considerable greater than the rate in Bangkok in 1966 (3.9%) and comparable to the mortality rate of 12.8% in Bangkok in 1958.

In addition to the Saigon-Cholon area, hemorrhagic fever was reported in the following provinces of South Vietnam: Gia Dinh, Bien Hoa, Long An, Long Khanh, Binh Duong, Binh Long, Hua Nghia, and Phoc Tuy.

Hemorrhagic Fever on Koh Samui:

Koh Samui is the main island in a small archipelago located in the gulf of Thailand, about 9° North of the equator. (Figure 3). The nearest mainland port is 5 hours away by boat.

Prior to December 1965, the island's two physicians had not seen hemorrhagic fever among the populace. Both men were familiar with the disease, and readily recognized its appearance in December 1965, when 20 cases, with 1 death, occurred in Ang Tong. On being informed of this outbreak, a team was sent to carry out a preliminary serological and entomological survey on the island. The diagnosis of dengue hemorrhagic fever was serologically confirmed, a high prevalence of dengue antibodies was found in a sample of children, and dengue—1 virus was isolated from one patient. *Aedes aegypti* were present in large numbers.

It appeared likely that a major epidemic of hemorrhagic fever might occur during the next monsoon season and preparations were made to study it should it occur. The anticipated epidemic began in July 1966 and lasted into October. A team from SMRL was present on the island throughout this time. The epidemiologic studies are described below.

Materials and Methods.

Description of the area. Koh Samui is about 12 miles long and 10 miles wide, with an area of almost 90 sq. mile. The population is approximately 25,000. The remoteness of the island, while rendering

study difficult from the point of view of access, was an advantage in that it limited the mobility of the population, particularly the age group in which we were most interested. Most of the population lives on the coastal plain of the island, which ranges in width from 2 to 4 miles. The center of the island is mountainous, the highest peak reaching over 1800 feet. Except in the mountainous center, the dominant form of vegetation is the coconut palm (*Cocos nucifera*); copra production is the economic mainstay of the island. Piles of coconut shells and husks are found in and around every village and scattered over the entire coastal plain.

The climate of Koh Samui is dominated by the tropical monsoon. Mean daily temperatures vary from 26° to 29°C throughout the year. Mean humidity ranges from 76 to 88%. During the dry season, from January through April. monthly rainfall averages 13-68 mm. The southwest monsoon (May-September) brings 135-170 mm of rain per month, and the Northeast monsoon season (October through December) has up to 300 mm of rain per month.

Clinical and Virologic Methods. All patients were seen by one of us (S.N.) who was the only practicing physician on the island at the time. A case record was kept of each patient seen in the course of the study. This record included name, age, sex, place of residence, date of onset, initial signs and symptoms and a careful description of the clinical course. Blood was obtained from each patient at the time of initial interview; this was usually within 3 days of onset of illness. A convalescent blood specimen was usually obtained 10-14 days after the acute specimen. Following clot separation and centrifugation, sera were stored on dry ice for shipment to Bangkok. Serum pairs were identified only by code number; serological and virological studies were carried out by personnel having no access to clinical records.

Each serum was acetone extracted and tested for hemagglutination-inhibiting (HI) antibody against dengue-1, 2, 3 and 4, Japanese encephalitis, and chikungunya antigens. Virus isolation from acute sera was done by plaque method in LLC-MK_2 cell culture and plaque forming agents were identified by plaque reduction neutralization test using reference monkey antiserum to prototype strains.

Based upon completed clinical records, patients were categorized by syndrome without reference to serologic or virologic data. Clinical categories were determined using the diagnostic criteria and nomenclature suggested by the World Health Organization. Patients with fever alone were classified as undifferentiated fever (UF). Patients with febrile disease exhibiting a positive tourniquet test or a few scattered petechiae were considered as having dengue fever (DF) syndrome. Patients were classified as hemorrhagic fever (HF) cases if in addition to fever and positive tourniquet test they exhibited one or more of the following: extensive petechiae, purpura, ecchymoses, epistaxis, hematemesis, hematuria or melena. Patients with shock (S), defined as pulse pressure less than 20 mm Hg., or evidence of a fall in systolic pressure below 90 mm Hg., were originally considered as a separate subcategory of the HF group, without reference to the presence or absence of hemorrhagic manifestations, for reasons discussed below.

The serologic response of each patient was independently classified by the following criteria: serum pairs which showed no detectable HI antibody, or no rise in titer from acute to convalescent were considered negative. A response which consisted of absence of antibody in the acute serum and a four-fold or greater rise in titer in the convalescent serum was designated a primary response. Demonstrable antibody in the acute specimen obtained within 3 days of onset of illness, plus either isolation of virus from the same specimen or a four-fold rise in titer to 1:640 or greater was taken as evidence of a secondary type antibody response. Specimens which showed antibody in an acute specimen obtained 4 or more days after onset plus either virus recovery or with a four-fold rise in HI titer were considered to be positive, but undifferentiable into primary or secondary response. Sera were rejected as incomplete if one serum of a pair was missing or if volumes were not sufficient to complete serologic examination.

At the completion of the study comparisons were made of clinical and serological classification of all patients.

Results.

A total of 148 individuals with 150 illnesses were included originally. From these, 139 complete adequate serum pairs were available for serologic and/or virologic study. The remaining 11 illnesses, for which complete serum pairs were not available, were excluded from further consideration. Table 3 presents the distribution of the 139, by admission clinical diagnosis and serologic response. Note that two individuals are represented twice, each having two episodes of undifferentiated fever (UF) at least one month apart. Since in both cases dengue was not implicated in the first episode of UF it was considered that they remained at risk; in one case the second UF was not dengue related, but in the other instance dengue virus was isolated from serum collected during the second UF episode. Therefore, they are treated as four separate cases.

Ninety illnesses were shown to be associated with dengue, in 21 cases dengue virus was isolated from the acute serum specimen. The additional 69 cases were associated with dengue on serologic grounds alone. Since no other agents were incriminated in more than 2 cases, we felf justified in considering this a dengue epidemic.

Figure 3 presents the geographic distribution of the 90 cases of dengue-associated disease, with the earliest date of onset recorded for each affected village. Figure 4 is the distribution of cases by week of onset. The disease first appeared on 10 July on the north coast of the island, later being reported from scattered villages over virtually the entire island. The last known case occurred on 5 October. The 3 week first "peak" in the epidemic curve (Fig. 4) coincides with the epidemic in and around the village of Mae Nam. A survey of the 87 households in this village indicated there were 224 children under age 15 living in Mae Nam. Thirty two become ill with dengue-associated disease during the epidemic; of these, 7 had dengue hemorrhagic fever with shock syndrome and 14 had hemorrhagic fever without shock. Thus, in a period of less than 2 months 9% of the children of Mae Nam had HF. An additional 5% were ill with DF or UF for a total dengue attack rate of 14.3%. Three other severe HF cases, with 1 fatality (presumably due to shock) had occurred in Mae Nam at the onset of the epidemic, before the arrival of the investigating team; these are not included in the data presented.

Considering the population age 15 and under at risk, the estimated dengue attack rate for the island as a whole was approximately 0.75% and in no village except Mae Nam was it over 2%.

The second "peak" in the epidemic corresponds to the simultaneous appearance of the disease in several scattered locations south of Ang Thong, primarily in Lipanoi and Talingngarm. No consistent pattern in the appearance of the disease in the scattered villages is apparent. The dates of onset do not seem to correlate with distance by road from the area on the north coast where the epidemic was centered. Table 5 and Figure 5 present the age and sex distribution of the 90 cases of dengue. The youngest child affected was one year of age, the eldest, fifteen. The median of the distribution is 6.6 years, the mode 7 years. Median and mode did not vary with sex, and since only 9 individuals exhibited primary antibody response, comparison by serologic classification is not revealing. When cases of DF and UF are combined, and their age distribution compared to that for HF and shock cases (Figure 6) the median (6.4 years) and mode (6 years) are lower than those for the HF age distribution (median 7.3 years, mode 7 years). The female preponderance seen in Table 5 (52:38) is more apparent if HF cases alone are considered (20:11). However, these ratios are not significantly different from the ratio of 1:1 which holds for the island population under age 15.

Dengue viruses were isolated from the acute serum of 21 patients, an isolation rate of 23%. Of these, 15 have been identified as to serotype. Three serologic types were represented among the 15 strains. The lack of relationship of virus type to clinical syndrome is shown in Table 6a. Dengue-2 and dengue-3 were both associated with shock cases as well as mild illness. The single dengue-1 strain came from a case of dengue fever syndrome.

Geographic and chronologic distribution of the dengue strains isolated from human cases are listed in table 6b. Dengue-2 and dengue-3 were widespread over the island during the period of the epidemic.

Discussion

The observations made from clinical and serologic studies during this epidemic indicate the difficulty of diagnosing disease due to dengue virus solely on clinical grounds. Undifferentiated fevers, dengue fever syndrome, and hemorrhagic fever were sometimes due to causes other than dengue virus infection. Considerable caution, therefore, is necessary in interpreting epidemiologic data from "hemorrhagic fever" epidemics in which the diagnosis is based solely on clinical grounds. Meaningful patterns can be obscured by the presence of other agents producing similiar syndromes. Of interest is the fact that no cases of chikungunya infection were seen in contrast to Bangkok epidemics where as high as 10% of HF cases (all mild illnesses) were due to chikungunya.

In this series 64 cases of UF were seen; only 35 (55%) were due to dengue. Of 36 cases of "dengue fever" syndrome, only 24 (68%) were shown to be dengue-caused. "Hemorrhagic fever syndrome" (i.e. without shock) was no more specific. Only eighteen (69%) of 26 cases were dengue-associated. The thirteen cases which exhibited shock, on the other hand, were all dengue-associated. The number is small, so that generalization with a high degree of confidence is not possible, but the data support the suggestion made elsewhere that the shock syndrome seen in connection with hemorrhagic fever epidemics is specifically associated with dengue. Dengue shock syndrome, or dengue hemorrhagic fever with shock, is the only segment of this clinical spectrum that can be differentiated by clinical examination with reasonable precision.

It is probable that all cases of hemorrhagic fever which occurred on the island during the epidemic are represented here. As has been noted, only one medical clinic was in operation during the epidemic, and all the cases included were treated at this central location. In addition, periodic visits were made to outlying areas by the attending physician as well as by members of the SMRL team, and no additional cases were found or reported. The island population is fairly closely knit; they were familiar with hemorrhagic fever from outbreaks which had occurred elsewhere in Thailand, and were generally sympathetic to the work of the investigating team so it is unlikely that any cases were concealed from or missed by the investigators. However, in general, no concerted effort was made to include all undifferentiated febrile illness, so this category (UF) represents an unknown but probably small proportion of febrile illness which occurred on the island during the time of the study.

Previous studies in Bangkok indicated that "cases tended to occur multiply in households". In an area when the "primary attack rate" was 3.6%, it was noted that of 271 families with at least one case of HF., 35 (13.2%) had two or more cases. This however does not indicate that these two rates bear a relationship analogous to primary/secondary attack rates, and cannot be used as evidence that siblings of cases are at higher risk than children in general. In the Mae Nam study where denominator data were available, it has been pointed out that the overall attack rate was 14.3%. Thirty two cases occurred in 22 households, having a child population of 82. If the first case in a household is considered the index case, then 60 siblings were at risk. Of these 10 had clinical illness; the attack rate among siblings of index cases was thus 16.7%, not strikingly different for that of the population as a whole. Thus the "clustering" in families appears to be a chance phenomenon entirely.

The HF cases in this epidemic appear to be generally older than has been reported elsewhere in Thailand or in Vietnam, and similiar to the ages reported in Malaysian epidemics.

The fact that all dengue shock cases did not exhibit hemorrhagic manifestations has been alluded to above. In this series, 9 cases with shock had hemorrhagic signs consistent with a diagnosis of hemorrhagic fever syndrome had shock been absent. However four cases, including the only fatality, had only fever and a positive tourniquet test or a few scattered petechiae as sole concomitant evidence of infection. If the "dengue shock syndrome" was an extreme manifestation of the same mechanism which produced the dengue hemorrhagic fever syndrome (i.e. the extreme end of the dengue infection spectrum) then all shock cases would be expected to exhibit severe hemorrhagic signs. This does not appear to be the cases; but neither

do the data suggest that shock is entirely independent of other symptoms; rather that the dengue shock syndrome is produced by a mechanism related to, but not necessarily identical with that resulting in hemorrhagic diathesis.

The data were examined to compare other characteristics of the group of dengue shock syndrome with those of cases of hemorrhagic fever syndrome without shock. Shock cases occurred throughout the epidemic and no evidence of geographic clustering was seen. Age distribution data (Figure 6) indicate that the average (median) age of HF cases may be higher than that of shock cases. Dengue viruses type 2 and 3 were isolated from shock cases, providing further evidence that the antigenic type of the infecting virus is not the sole determinant of the infecting virus is not the sole determinant of the production of the shock syndrome.

The hemorrhagic fever syndrome has been observed to result from an initial dengue infection; on Koh Samui one individual with a primary type antibody response to dengue had a clinically typical example of the syndrome. In contrast none of the thirteen shock cases were accompanied by primary type antibody response. Nine cases had secondary type antibody response and four were serologically positive for dengue, but response could not be classified with complete assurance as either primary or secondary, due to delay in obtaining acute specimens. In these four, initial serum specimens had high titers which probably indicate secondary-type serologic response.

The observations made thusfor support the hypothesis that dengue shock syndrome is produced by a specific immunologic phenomenon elicited by a second, heterotypic, dengue infection occurring a certain critical period of time following an initial dengue infection. The initial, or primary, dengue infection may produce a mild form of hemorrhagic fever but either undifferentiated fever or dengue fever syndrome are more commonly produced. Shock does not occur. Following recovery, patients have immunity to heterologous dengue infection for a variable period of time (of the order of 6 months). During this time "sensitization" may be occurring in certain individuals, depending upon host factors. Following the immune period, in "sensitized" individuals, there occurs a period where a second, heterotypic, dengue infection results in development of shock syndrome and/or hemorrhagic fever syndrome, the period of susceptibility for the latter probably extending beyond that for the former syndrome. Thus a critical "configuration" of circumstances, age at initial infection, interval between infections, and possibly also dengue type and dose, are necessary to produce the dengue shock syndrome.

The finding on Koh Samui of three distinct dengue serotypes (dengue-1, 2 and 3) present during the epidemic is consistent with the previous findings that in areas where dengue hemorrhagic fever is prevalent (e.g. Bangkok, Manila, Singapore and Saigon) three or four dengue serotypes have been found.

Until it is possible to directly demonstrate the pathogenic mechanism (s) of shock syndrome in man or suitable animal models, it is necessary to continue careful and detailed studies of epidemiologic patterns manifested by this imperfectly understood disease.

<u>Entomologic Studies on Koh Samui.</u> The initial SMRL survey on Koh Samui in February 1966 established that both <u>Aedes aegypti</u> and <u>Aedes albopictus</u> were present on the island. <u>Aedes aegypti</u> was subsequently found to be widely distributed throughout the island, both in villages and in isolated farmhouses, and during both the rainy season (May–December) and dry season (January–April) (Table 7a).

During November a survey of larval habitats of <u>Aedes aegypti</u> and <u>Aedes albopictus</u> was carried out in the vicinity of homes of September cases of THF in tambons Mae Nam and Taling Ngam on the island of Koh Samui. The frequent occurrence of <u>albopictus</u> larvae in artificial containers (e.g., water jars, gasoline drums, plant containers, etc.) in close proximity to houses (inside a house on one occasion) and sharing of the same habitat with <u>aegypti</u> larvae was observed (Table 7b). During this same period adults of both species were frequently collected resting and biting inside houses, however <u>aegypti</u> adults were collected biting outdoors on only one occasion (Table 7c).

During the course of the 1966 HF epidemic both *A. aegypti* and *A. albopictus* adults were collected from the vicinity of the dwellings of recent cases of the disease for virus isolation attempts. Nine strains of dengue virus were isolated from a total of 122 *A. aegypti* collected from houses in Ang Thong and Mae Nam in July. Four additional isolations were obtained from a total of 1110 *A. albopictus* collected during September. The occurrence of these two vector species together in the presence of this dengue outbreak is strikingly similar to the situation in Saigon and Singapore and quite dissimilar to that of Bangkok (and probably Manila) where only *aegypti* appears to be involved in dengue transmission.

In February 1967 collections of *A. aegypti* and *A. albopictus* were made from several areas on Koh Samui, from Koh Pangan (10 kilometers north of Koh Samui) and from Koh Paluai (25 kilometers west of Koh Samui) for purposes of insecticide susceptibility studies. All houses visited on these smaller, more isolated islands were infested with *A. aegypti* (22/22 on Paluai and 25/25 on Pangan), but there was no evidence that *A. albopictus* was present although the ecological conditions there apparently were similar to those on Koh Samui. The apparent absence of *albopictus* on these outer islands may have been due to a decline in its population caused by the dry season, for *albopictus* was found with difficulty in areas of Koh Samui where during the previous November it had been abundant. Susceptibility tests of larvae of *A. aegypti* from Ang Thong and Taling Ngam on Koh Samui and from Koh Paluai and Koh Pangan indicated that this species has become resistant to DDT and dieldrin in those areas (details of these tests given under the report on mosquito studies). Results of tests of the insecticide susceptibility of *A. albopictus* from Koh Samui are not yet available.

SUMMARY

Dengue hemorrhagic fever continues as a major public health problem in the pediatric age group in Bangkok and many other areas in Thailand. In 1966 a further spread of this disease was documented in the city of Udorn Thani in northeast Thailand.

Epidemic dengue hemorrhagic fever occurred in Saigon in 1966 with a peak incidence during the monsoon season in June and July. The age distribution in Saigon is similar to that seen in Bangkok. However, the reported mortality rate is considerably higher.

Epidemiologic studies were carried out during an epidemic of dengue hemorrhagic fever which occurred on the island of Koh Samui between 10 July and 5 October 1966.

Title: Arbovirus Infections in Men and Experimental Animals

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Objectives:

a. To develop improved virologic and serologic methods for diagnosis and study of arbovirus diseases in Thailand.

b. To determine the antigenic relationships and study the biologic properties of arboviruses of medical importance.

c. To obtain information on the sequential changes in the immunoglobulin response of man and experimental animals to single and multiple dengue infections and to relate these observations to disease processes.

d. To study the etiology of human cases of encephalitis and determine the importance of arboviruses and other viruses as causative agents.

Description:

The plaque reduction neutralization test (PRNT) for dengue virus antibody described in the previous annual report was further investigated to determine the effect of varying conditions and to estimate the accuracy of the test. The rate of thermal inactivation of several dengue virus strains was determined. Serum from human and animal sources were tested to determine the amount of heat labile inhibitors present. A statistical analysis of tests performed with antiserum of known comparative potency was done to determine the accuracy of the test for estimating antibody levels.

A method of primary isolation of arboviruses from serum and mosquito pools was developed using a plaque assay in LLC-MK_2 tissue culture. The newly developed method was compared with intercerebral inoculation of suckling mice for sensitivity to unpassaged virus strains.

Dengue viruses isolated from a variety of sources including dengue fever cases, hemorrhagic fever cases, and mosquito pools were identified by the plaque reduction neutralization test method using reference monkey antiserum.

Autigenic relationships of several strains within each serotype were compared.

Experimental dengue infection in monkeys and gibbons were studied to determine susceptibility to subcutaneous infection, duration of viremia, and immune response.

Using density gradient centrifugation, ion-exchange chromatography and gel filtration, studies were done to determine the nature of the antibody response to both primary and secondary dengue infections in man. Immunoglobulins were identified by immunoelectrophoresis and immunoprecipitin methods; antibody activity was tested by hemagglutination-inhibition and complement-fixation.

Cases of human central nervous system disease referred from hospitals in various areas of Thailand were studied by serologic and virologic methods to determine the viral causes of humon CNS disease in Thailand.

Progress:

Plaque Reduction Neutralization Test.

The method for performing the PRNT for dengue neutralizing (N) antibody in LLC-MK_2 cell culture was described in detail in the previous annual report. A serum-virus incubation time of 1 hour at 37°C is routinely used. Since heat lability of seed virus is an important factor which affects the amount of non-viable antigen in the test system, several experiments were performed to determine the effect of temperature on dengue virus seeds diluted in M-199 containing 5% inactivated calf serum buffered with $NaHCO_3$ to pH 8.2. This approximates closely the conditions of the neutralization test. Virus suspensions containing 50 to 100 pfu at zero time were heated to 37°C or 42°C in water baths and infectivity determined at various time intervals by plaque assay. The following strains were tested at indicated suckling mouse passage level:

Hawaii (dengue-1)		sm 125
TH-Sman	"	sm 4
# 10572	"	sm 3
New Guin. "C" (dengue-2)		sm-26
TH-36	"	sm-14
# 10286	"	sm-4
H-87 (dengue-3)		sm-21
H-241 (dengue-4)		sm-25

Results expressed as percent survival are summarized in Table 8. A marked difference between dengue strains is apparent. TH-36 was the most heat labile of the strains tested showing only 28% survival after 1 hour at 37°C. Hawaii and No. 10572 were somewhat less labile, however, the reduction of infectivity after 1 hour at 37°C was significant. H-87 appeared least affected by heat and the remaining strains were moderately heat resistant.

Heat resistance varies markedly between strains of dengue viruses. It apparently is not related to mouse passage and probably is not related to antigenic type. Three of the 8 strains tested showed a 50% or greater loss of infectivity after 1 hour at 37°C.

The plaque reduction neutralization test is very sensitive to the effect of non-specific anti-viral substances in serum. Sera from several species were tested against dengue viruses by plaque reduction to measure non-specific anti-viral activity. The tests were performed in the identical manner as the PRNT except the sera were freshly collected, had never been frozen, and were not heat-inactivated. Heat

inactivated (56°C, 1 hour) sera were used as controls. Results, given in Table 9, show the presence of non specific inhibitors in all sera tested. In the majority of sera significant anti-viral activity was present at the 1 : 10 dilution. The common occurrence of heat labile non-specific anti-viral substances in fresh sera precluded the use of fresh sera as a source of "accessory substance" to enhance virus neutralization.

To determine the accuracy of the plaque reduction neutralization test when two or more sera are simultaneously tested against a single virus for purposes of determining comparative potency, the following experiments were performed: Dengue-1 (Hawaii) antiserum was diluted 1 : 4 and 1 : 8, the two dilutions were then treated as serum of unknown potency and tested against the homologous Virus. In a second test dengue (TH–Sman) antiserum was diluted 1 : 4 and 1 : 8 and tested in parallel with undiluted serum. The dilution factor was unknown to the technician performing the test who treated each one as a whole serum of unknown titer. The results obtained are presented graphically in figures 7 and 8. The 50% effective dose (ED_{50}), 95% confidence limits of the ED_{50}, relative potency of the two sera in each test, and the 95% confidence limits of the relative potency were calculated by method for parallel-line, graded response bioassay. Results of statistical analysis indicated that in both tests the 95% confidence limits of the ED_{50} were exclusive, indicating that this technique can distinguish two fold differences in 50% plaque reduction titers. In addition the dose response curves were linear and parallel indicating that estimation of 50% plaque reduction end points by the method of Cutchins is in an appropriate way of describing potency of antisera.

Additional experience with the use of this test for comparing serum titers against different virus strains, and for comparing results of tests performed at different times with different lots of seed virus indicates that, under conditions where such additional variables are present, two fold differences may not be significant. This is especially true with early antiserum where the sloe of the dose response curve is steep. In such cases, observed differences in 50% plaque reduction titers must be 4 fold or greater to be certain of biologic significance.

Dengue Virus Identification.

The PRNT used in conjunction with reference anti sera made by a single subcutaneous injection of live virus in *Macaca irus* monkeys has proven to be an excellent method for identification of dengue viruses. Dengue virus strains identified by this method are listed in Table 10 with host and passage level, country of origin, and year of isolation. The reference strains were originally obtained from Dr. Wm. McD. Hammon. The Thailand and South Vietnam strains were isolated in this laboratory from human serum. Twenty-five were isolated in suckling mice, nine were isolated in BS-C-1 tissue culture by the challenge virus resistance method and three were isolated by direct plaque method in LLC-MK_2 cell cultures.

The Thailand viruses from 1962, 1963 and 1964 are selected strains that could not be readily identified by complement fixation tests or neutralization tests in BS-C-1 tissue culture. Thus they are not representative strains from that period. After 1965, identification by other methods was not done.

The majority of the strains, including all four serotypes, were isolated from patients with the clinical diagnosis of hemorrhagic fever. Three strains came from patients with an undifferentiated febrile illness and thirteen strains were isolated from cases of dengue fever in caucasians.

The Pak-18 strain was obtained from WRAIR, and the Philippine strains were isolated in this laboratory by Dr. Basaca Sevilla.

The Tahiti strain was isolated from human serum by Dr, Leon Rosen, during the dengue epidemic in late 1964 and was sent to this laboratory for identification. This agent (T 502066) was isolated in mice by blind passage but produced no symtoms in mice up through 10 passages. Virus growth was identified by resistance of mice to challenge with virulent dengue virus and by production of challenge virus resistance on passage to BS-C-1 cell culture. BS-C-1 passage virus was propagated in this laboratory in LLC-MK_2 cells.

Antisera to all strains were made in Macaca irus monkeys by a single subcutaneous injection of 10^3 to 10^4 plaque forming units (pfu) or, in the case of the mouse adapted reference strains, 10^4 to 10^5 suckling mouse intracerebral LD_{50}. Serum was harvested approximately one year post inoculation for the reference strains and 2 months post inoculation for the other strains. Mouse antiserum to dengue-4 was made by three subcutaneous injection of 10^5 suckling mouse IC LD_{50} one week apart and harvesting serum after 4 weeks.

The results of cross neutralization tests with reference dengue strains and antisera are given in Table 11. The neutralizing antibody titers clearly distinguished between types 1 through 4. TH-36 cannot be clearly differentiated from dengue-2 nor can TH-Sman be clearly distinguished from dengue-1 although small differnces in neutralization titers are observed.

The type 1 and type 2 antisera reacted only within their serotypes with no detectable titer to types 3 or 4. Antisera to types 3 and 4, on the other hand, gave evidence of cross reaction with heterologous types. However, wide differences between heterologous and homologous titers were observed. The most notable cross reaction was the titer of 1:90 of dengue-3 antiserum against type 1 virus compared to a heterogous titer of 1:350. This cross reaction is not reciprocal and does not preclude differentiations between viruses within the 1 and 3 serotypes.

Results of testing 10 virus strains in the type 1 group, including strains from Thailand and South Vietnam against the reference antisera are given in Table 12. In addition, monkey antisera to four Thai strains was tested. All of these agents were neutralized by dengue-1 and TH-Sman antiserum. The titers obtained by testing these isolates with dengue-1 antiserum were uniformly lower than the homologous serum titer. The titers against the TH-Sman antiserum, however, were not significantly different from homologous titers. Three strains showed very low titered cross reactions, of doubtful significance, with dengue-2 or TH-36 antiserum. Seven of the ten strains were neutralized in low titer with the dengue-3 antiserum. Antisera made to the 4 Thai strains neutralized TH-Sman virus with titers not significantly different from homologous titers. There appeared to be some variation in neutralization of dengue-1 virus by the Thailand strain antisera. Antisera to No. 12900 and 22448 had similar titers against homologous virus dengue-1 and TH-Sman. However, antisera to strains Nos. 14580 and 18280 had somewhat lower titers to dengue-1 than to TH-Sman or homologous strains.

Eight dengue-2 strains were included in this series and all were neutralized in high titer by dengue-2 antisera and in very low titer by dengue-3 antisera as shown in Table 13. The titers of dengue-2 antisera against the Southeast Asian strains were significantly lower than against the homologous virus and against TH-36, both of which are highly mouse adapted.

Table 14 shows the results of neutralization tests with 14 strains which were neutralized by type 3 antisera, with one exception, a marked antigenic uniformity among dengue-3 group is apparent. None of the dengue-3 strains reacted with heterologous antiserum. Antisera made against strain No. 14670 and the Pakistan-18 strain reacted similarly with the homologous virus and with the reference dengue-3 strain. The Tahiti strain was neutralized only by type 3 antiserum but the serum titer was significantly lower than titers to all other strains.

The monkey antiserum made against the dengue-4 prototype strain had comparatively low titer compared to mouse antiserum to the same virus as seen in Table 15. The monkey antiserum to strain 14486 had a similar low titer, however, there was no cross reaction with the other serotypes. As shown in Table 16, 8 isolates were neutralized by dengue-4 mouse antiserum although five of the eight strains reacted with lower titers than the prototype strain. The cross reactions noted between dengue-4 prototype and 14486 indicate some degree of antigenic variation within the group.

The results described above indicate that the plaque reduction neutralization test is an extremely useful method for identification of newly isolated dengue virus strains. Of primary interest is the fact

that strains isolated in tissue culture could be identified without adaptation to mice and without extensive adaptation to a tissue culture system. It was possible to identify several strains of dengue-3 and dengue-4 which could not be identified by mouse neutralization test, complement fixation tests, or neutralization tests in BSC-1 tissue culture. In our experience a tissue culture seed with a titer of 500 plaque forming units per 0.15 ml is adequate for accurate typing by this method.

Monkey antisera prepared in Macaca irus monkeys by a single subcutaneous inoculation of virus were shown to have a high degree of specificity and this presently is considered to be the method of choice for producing dengue antiserum. When tested by the plaque reduction neutralization test sufficiently high titers are observed to identify viruses and measure antigenic relationships.

It is of interest that all viruses tested could be placed within one of the 4 major dengue serotypes by a neutralization test against antisera to reference strains. In all cases where antisera to new isolates were made, cross neutralization tests confirmed the results obtained by the typing test. These results suggest that the typing test using reference antisera is sufficient for routine epidemiologic studies. Antigenic variation within the major serotypes has been previously emphasized by Hammon, and the results described above indicate antigenic differences between dengue-1 and TH-Sman and dengue-2 and TH-36 as well as similar within the dengue-4 serotype. On the basis of these results, however, variation does not appear to be of sufficient magnitude to justify classification of other than 4 major serotypes.

There appeared to be no significant differences between virus strains isolated from hemorrhagic fever patients and those isolated from patients with undifferentiated fever and classical dengue. The two strains of dengue 4 isolated from patients in South Vietnam represent the first strains of this serotype found in that area.

Identification of Dengue Viruses from Koh Samui.

The identification of dengue viruses isolated from human sera and mosquitoes collected on Koh Samui are presented separately below because different lots of typing antisera were used and Some special problems arose. All viruses were isolated in LLC-MK_2 cell culture and the mosquito strains were also reisolated in suckling mice.

Results of identification of 16 strains isolated from human sera are presented in Table 17. The six type 3 strains appear similiar to previously identified type-3 strains from Bangkok and other areas. Several of the type 2 strains, especially 24367, 24453, 24464, 24742 and 25076 were neutralized only by low dilutions of the dengue-2 (New Guinea "C") antiserum.

Similiar results were seen when tissue culture seeds of viruses isolated from mosquitoes were tested against reference antiserum. Table 18 gives the results of typing by plaque reduction neutralization test of 7 dengue strains all of which are dengue-2. Of interest is the fact that suckling mouse passage BKM 551-66 was neutralized by high dilutions of dengue-2 antiserum whereas the tissue culture line of the same virus was neutralized only by low dilutions of the same antiserum.

Antisera made in monkeys to suckling mouse and tissue culture strains of BKM 551-66 both had low neutralizing antibody titers when tested against the tissue culture propagated virus; however the antisera made to suckling mouse propagated virus neutralized prototype high mouse passage dengue-2 strains and the homologous virus at high titer.

This phenomenon is unexplained and introduces additional difficulties in identification of some newly isolated dengue viruses. The effect of the host on the susceptibility of dengue virus strains to neutralization by antibody is presently being investigated.

Virus Isolation Methods.

Isolation of arboviruses had previously been attempted in suckling mice and BS-C-1 cell cultures. The challenge virus resistance technique using BS-C-1 cells proved to be extremely sensitive for dengue and Japanese encephalitis viruses. As with suckling mice, however, this method often requires up to three blind passages (with attendant dangers of cross contamination) before virus presence can be detected. A large number of tubes is also required, which limits the number of specimens which can be carefully tested. A system sensitive to all arboviruses known to be present in Thailand but which would be simple, direct and suitable for testing a large number of specimens was desired.

Because all known Thai arboviruses produce plaques in $LLC\text{-}MK_2$ cell cultures, this easily handled cell line was adopted for the isolation system. At present, isolation from all non-blood-engorged mosquitoes are being attempted by direct plaques. Mosquito suspensions undiluted and diluted 10 and 100 fold are adsorbed onto $LLC\text{-}MK_2$ cell monolayers in 1 oz bottles and overlayed with agar as previously described. In addition, two bottles are inoculated with undiluted mosquito suspensions and held as fluid cultures for virus seeds. Agar overlayed bottles are reoverlayed and stained at 7 days and examined on days 8 to 12. If plaques appear the fluid cultures are harvested on day 14 and titered. If the titer is not adequate for plaque reduction typing, additional passages are made.

For blood-engorged mosquitoes and tissue or serum, where antibody may be present, a direct and delayed plaque system was developed as follows:

Specimen for Virus Isolation

Undiluted	Undiluted	Undiluted and 1:25
Fluid Culture	Fluid Culture	Plaque Cultures
(1) If direct plaque+ harvest on day 14 (2) If delayed plaque+ harvest no sooner than day 14. Pass if necessary	Adsorb 2 hrs Maintenance medium for 7 days, then add agar overlay	Add agar overlay after 2 hour adsorbtion.
Seed Virus	Delayed Plaques	Direct Plaques

The delayed plaque system allows time for (1) virus—early antibody dissociation; (2) adsorbtion when very small numbers of viruses are present and (3) one cycle of replication to occur. The delayed plaque system is more sensitive than direct plaques alone, and has resulted in isolation of about one third more dengue viruses (Table 20).

For isolation of dengue virus from mosquito suspensions, however, the $LLC\text{-}MK_2$ plaque system appeared to be superior to suckling mice, the mice either failing to detect virus or being resistant to dengue 2 challenge but not becoming ill. (Table 22). It should be noted, however, that it has been difficult to prepare virus seeds of high titer from some mosquito isolates.

Suckling mice appear to be fairly sensitive for isolation of dengue viruses from human serum. Mice were successfully used to isolate viruses from 15 plaque positive viremic human sera from the Koh Samui and Vietnam dengue outbreaks. These sera contained dengue types 1, 2 or 3. In 9 of 15 cases, however, mortality did not occur until the third blind passage (Table 21), whereas in the direct-delayed plaque system virus is detectable in the first passage.

Characteristics of dengue virus growth in LLC-MK$_2$ cells

Use of LLC-MK$_2$ cells for detection of dengue viruses in acute phase human sera and in suspensions of field-collected mosquitoes has been extremely successful. There has been some difficulty, however, in consistently making cell culture virus seeds with titers adequate for typing largely because the optimal time from inoculation until harvest was unknown. Experiments designed to determine this interval in LLC-MK$_2$ tube cultures of various ages, inoculated with minimal doses of low tissue culture passage dengue-2 virus, were carried out. At the same time, similar aged tube cultures of primary human embryonic lung and kidney cells were inoculated in an attempt to determine if diploid human cells supported dengue virus replication to higher titer than did the continous monkey kidney cell line.

In addition to LLC-MK$_2$ monolayers from one through seven days old at the time inoculation, cell suspensions were also inoculated with virus. Cells were counted daily before new cultures were prepared so that on the day of virus inoculation the cell populations of each age group were approximately equal. Human primary cells were of limited availability and so only monolayer tube cultures seven days of age were used. The cells were washed once with Hank's balanced salt solution (HBSS) and inoculated with 15-20 pfu of dengue-2 virus, BKM-540 in LLC-MK$_2$ passage two (first experiment) and passage three (second experiment). The virus was allowed to adsorb at 37°C for 1 1/2 hours. The tubes were then maintained with M-199 with 5% heat inactivated calf serum, pH 8.3. Cell-associated virus was harvested in 1 cc per tube of medium 199 with 50% heat-inactivated calf serum. In the first experiment (cell suspensions and monolayers 1-4 days of age) the virus was harvested from randomly selected tubes on alternat days by mechanically lysing them in the micro-homogenizer attachment of the Omni-Mixer, with sterile sand at about 30,000 rpm for three minutes. This method provided lysis superior to sand alone with vortex mixing or osmotically shocked cells followed by sand and vortex mixing, and equal to three cycles of rapid freezing and thawing. Since freezing and thawing was the simpler method, it was used for harvesting dengue virus in the second experiment. Cell lysates were centrifuged and stored at-70°C until assayed.

Ability to support virus replication increased with the age of the cell monolayer at time of inoculation up to four days of age, after which there was little difference. Virus titers after 19 days incubation, however, were higher for 7 day old cells than the younger 4 and 5 day old cells. Virus titers were independent of the slight differences in cell populations. Virus titers in LLC-MK$_2$ cells rose to a peak on day 6 (second experiment) and day 8 (first experiment) fell two days later and rose to a new, higher peak four to 8 days after the first peak (Figures 9 and 10).

The occurrence of both peak titers in the second experiment two days in advance of and higher than of the first experiment suggests that the one addition LLC-MK$_2$ cell passage may have resulted in further adaptation of the virus to the cell system.

The virus titers in human embryo kidney and lung cells (Figure 10) rose more slowly, the first peak occurring on day 8-10, the titer falling and then peaking again on day 16, and falling again on day 19, but at no time achieving the maximum titers of the LLC-MK$_2$ cells.

In both cell systems, the occurrence of two peaks in virus titers suggests that when the virus input is small, virus replication proceeds synchronously in the cells, mature virus appearing, reinfecting and eclipsing, and progeny virus maturing again, all at about the same time. It was not until after the second peak, 19 days post infection, that the synchrony broke down. This was most striking in the human

embryonic lung cells, when only one pfu was seen in three bottles on day 12, a drop in titer of over 10^3 pfu in 48 hours. These cycles point up the importance of allowing an adequate time interval between inoculation of virus and harvest.

Virus propagated in human embryonic lung cells produced plaques of uniform size and clarity. Both kidney cell types, human primary and monkey heteroploid, produced virus of mixed plaque size and morphology, small clear plaques and large hazy plaques in a ratio of about 3:1.

Dengue Viremia in Primates.

In the course of the studies on dengue antibody in monkeys, viremia was measured in 12 *Macaca irus* monkeys following a single subcutaneous inoculation of dengue virus. A variety of dengue viruses including high mousl passage prototype dengue-1 and low tissue culture passage local strains were used. The results, shown in Table 23, indicate that *Macaca irus* monkeys regularly developed viremia following inoculation with dengue viruses types 1 and 4.

A preliminary experiment in March 1966 indicated that the white-handed gibbon (*Hylobates lar*) developed HI antibody following subcutaneous inoculation with dengue-1 virus. To determine the usefulness of this species for studies of cross immunity of dengue virus, a second experiment was done in which 7 gibbons were given a subcutaneous inoculation of approximately 100 plaque forming units of a local dengue-2 strains (No. 1004+) in the 3rd BS-C-1 passage. Five gibbons had no previous exposure to dengue and two had had a dengue-1 infection 5 months previously. Viremia was estimated on days 2 through 10 following inoculation.

Viremia developed in all gibbons (Table 24). The five animals which had had no previous exposure to dengue to virus all had between 4 and 6 days of viremia with maximum titers of approximately $10.^{?}$ The two gibbons which had previous dengue-1 infections had a shorter viremia with lower titers.

It appears that both the cynomalogous monkeys and white-handed gibbons will be useful for future studies on cross protection between dengue strains. With the virus strains tested, all inoculated animals developed viremia. It is anticipated that detailed studies on cross protection between dengue virus strains will be carried out using these animals as experimental hosts.

Dengue Antibody and Monkeys.

Studies reported in the previous annual report demonstrated that experimental infection of *Macaca irus* monkeys with dengue-2 virus resulted in an early 19S antibody response followed by production of 7S antibody. The 19S antibody was identified by immunoelectrophoresis as Ig-M and the 7S antibody as predominately Ig-G. Ig-M antibody fell to undetectable levels within 2 months after infection. Further studies were carried out to determine the nature of the immunoglobulin response following primary and secondary dengue infections in monkeys. Two monkeys (A-21 and A-37), free of B group arbovirus antibody, were inoculated subcutaneously with 120 pfu of a local strain of dengue-2 virus in the 3rd tissue culture passage. Approximately one year later the same monkeys were inoculated subcutaneously with 1000 pfu of dengue-1 virus in 2nd tissue culture passage.

Table 25 presents the results of density gradient centrifugation of serum collected at frequent intervals following these experimental infections. In both animals the primary dengue-2 infection caused a 19S antibody response (measured by HI test) beginning on day 10 and disappearing prior to day 42, the 7S response began on the 12th day following infection and 7S antibody was present one year later. The second dengue infection with dengue-1 virus did not result in production of detectable amounts of 19S antibody; however, 7S antibody titers rose rapidly by the 12th day post inoculation.

The virus neutralizing properties of mercapto-ethanol sensitive 19S antibodies against dengue viruses had been previously demonstrated. The specificity of such antibody was compared with the specificity of 7S antibodies in sera from 2 monkeys 14 days following dengue-2 infection. Pooled fractions from 19S and 7S zones of the density gradient were extracted with acetone and tested by plaque reduction neutralization test against prototype dengue viruses. Results are given in Table 26. Monkey A-39 had a very high 7S antibody titer. The apparent minor differences in specificity between 19S and 7S fractions are probably due to relative amounts of antibody rather than actual differences in specificity.

Neutralizing antibody in whole serum of monkeys A-21 and A-37 was measured before and after the secondary dengue-1 infection by plaque reduction neutralization test. The results, given in Table 27, show type specific neutralization one year after the dengue-2 infection, but 21 day after the dengue-1 infection broad cross reactivity was apparent. The rise of neutralizing antibody (Table 28) roughly parallels in time the rise of 7S HI antibody in these monkeys, reaching high titers by 14 days post inoculation.

Immunologic Response to Dengue Infection in Man.

Preliminary studies reported in the previous annual report demonstrated a short period (2 to 4 weeks) of 19S antibody production following primary dengue infections in Thai children. The cases of secondary dengue infection studied, however, had little or no 19S antibody and a marked 7S antibody response. These studies were continued and a total of 23 sera from 8 cases of mild dengue infections exhibiting a primary type of antibody response were fractionated by sucrose density gradient ultra-centrifugation. The fractions were tested for anti-dengue HI activity and for sensitivity to reduction by 2-mercapto ethanol. A typical pattern of primary response is shown in Table 29. The 19S mercaptoethanol-sensitive, antibody appeared on the 6th day of illness and 7S antibody appeared on the 10th day. Similar patterns were seen in the other 7 cases studied with 19S antibody appearing as early as the 4th day of illness and persisting as long as the 30th day. In 3 cases, 19S antibody was no longer detectable in the serum after 18 days.

Forty-one sera from 14 cases of secondary dengue infections were fractionated. Table 10 shows a typical pattern seen in a secondary dengue infection associated with the dengue shock syndrome. A marked 7S antibody rise preceded 19S antibody formation, and the 19S response was markedly suppressed. In 5 of 14 cases, small amounts of 19S antibody were detected between the 4th and 18th day of illness, however, in the remaining 9 cases, no 19S antibody was found.

These studies utilizing density gradient ultra-centrifugation technique indicated that, in the case of primary dengue infections, the first anti-dengue immunoglobulins detectable are 19S globulins, and 7S antibodies appear 1-3 days after the appearance of the 19S antibodies. In such cases if antigen-antibody complexes are formed within the host the complexes would consist predominately of 19S antibody and viral antigen.

In the case of secondary dengue infections, the first detectable antibodies are 7S globulins which rise rapidly in titer; the 19S globulins in most cases cannot be detected or, are produced in small amounts. In the secondary cases, therefore, antigen-antibody complexes, if formed, would contain predominately 7S antibody.

Since patients with dengue shock syndrome have a secondary type antibody response which may be associated with the pathogenesis of this syndrome, studies were carried out to determine the identity of the 7S anti-dengue antibodies present in the serum during and after the shock phase.

Patients with hemorrhagic fever who were admitted to the Children's Hospital in the early stages of dengue shock syndrome, were carefully followed clinically, and blood specimens obtained at frequent intervals. Sera were quick frozen immediately after separation and stored at-70°C. Sera were tested for dengue HI antibody and pooled for fractionation by DEAE-cellulose chromatography.

Pooled sera (4 to 8 ml) were applied to a 2.2 x 40 cm column of Sephadex G-25 and eluted with 0.03 M phosphate buffer, pH 6.4. The protein containing eluate from the Sephadex column was applied to a 2.5 x 80 cm column of DEAE-cellulose previously equilibrated with 0.03 M phosphate buffer, pH 6.4. The protein was eluted from the column with 0,03 M buffer at a flow rate of 5 ml/minute. Protein concentration (optical density at 280 mu) was measured continuously and the protein containing eluate pooled as fraction I. Subsequent elutions were made in a similiar manner with 0.1 M and 0.3 M phosphate buffers also at pH 6.4. Three fractions were thus obtained and the globulins in each fractions were concentrated by precipitation in 50% ammonium sulphate. Precipitates were sedimented by centrifugation, dissolved in normal saline, and dialysed against normal saline for 24 hours.

The immunoglobulins contained in the concentrated fractions were assayed by two methods. Single radial diffusion in agar containing specific antibody against Ig-M, Ig-G or Ig-A was done using commercial reagents (Immunoplate, Hyland Laboratories). Immunoelectrophoresis was done using goat antiserum against Ig-G, Ig-A, and Ig-M (Hyland Laboratories), and antihuman globulin serum prepared in rabbits.

Each fraction was tested for HI antibody against 4 dengue antigens and the sensitivity of the HI antibody to reduction by 2-mercaptoethanol was determined.

The HI antibody titers of the sera and the serum pools are given in Table 31. In all cases high antibody titers were present at the time shock was observed. Table 32 presents the results of immunoglobulin and antibody assays of the fractions from the DEAE chromatography.

The radial diffusion method proved superior to immunoelectrophoresis for detection of low concentrations of immunoglobulins, and in addition, allowed measurement of concentrations.

The method used for fractionation was found to have two major disadvantages. First, the yield of partially purified immunoglobulin was somewhat low; due in part to loss on the DEAE column and in part to the method of concentration. Second, and more importantly, fraction II and III, containing Ig-A and Ig-M, respectively, always contained measurable amounts of Ig-G as well.

Fraction I, eluted with 0.03 M phosphate buffer, contained >95% of the total Ig-G, and no Ig-A or Ig-M was detectable in this fraction in any case. It is apparent from table 32 that most of the antibody activity was also found in fraction I in each case. HI antibody titers in fraction I are 40 to 1000 fold greater than the titers seen in fraction II or III.

Ig-A was found in fraction II although traces were present in fraction III in cases HFI-747 and HFI-749. Ig-M was detected only in fraction III.

It is apparent that the HI antibody activity in fraction I is related primarily to the Ig-G content. In every case, the HI antibody titers of fraction III were low and were not affected by treatment with 2-mercaptoethanol. It is reasonable to conclude, therefore, that the HI antibody activity in fraction III was due to the small amounts of Ig-G present and not due to Ig-M, which is sensitive to the action of 2-mercaptoethanol. This was confirmed in case HFI-773 by sucrose density gradient ultracentrifugation of the serum pool. The results, given in Table 33 show that no HI antibody activity was found in the Ig-M containing fractions (2, 3 and 4), and all HI antibody was 2-mercaptoethanol resistant. The findings in these cases are consistent with previous findings that little or no anti-dengue Ig-M antibody is present in the sera of patients with dengue shock syndrome.

The results obtained with the Ig-A containing fraction II were more difficult to interpret. The amount of Ig-G present in fraction II in each case was of the same order of magnitude as the amount of Ig-G in fraction III. The HI antibody titers are likewise in the same range. Allowing for the inaccuracies of measurement of both immunoglobulin concentration, and of antibody titers it appears that all HI antibody activity could be ascribed to the Ig-G content of fraction II in each case. However, it is impossible from these results to state with assurance that Ig-A did not contribute to the antibody activity measured. The amount of HI antibody activity due to Ig-A in the original serum pools, if present at all, must be very small in comparison with Ig-G antibody activity.

Additional studies of this nature are in progress as well as experiments to improve the techniques of purifying immunoglobulins.

Complement fixation by 19S and 7S antibodies to Arboviruses.

Studies reported above have shown that both 19S and 7S anti-dengue immunoglobulins react in the hemagglutination-inhibition (HI) test and neutralize dengue viruses in vitro. Little information is available however on the complement fixing (CF) properties of human immunoglobulins from sera containing antibodies against arboviruses. The fact that CF activity is low or absent during very early convalescence from a primary dengue or a chikungunya infection when both HI and N antibody activity are present and 19S globulins comprise the major portion of active antibody in the serum, suggests that the CF activity of 19S globulins is low. Studies were done to determine the CF activity of 19S globulins in vitro.

Using sucrose density gradient methods, partially purified preparations of 19S and 7S globulins were obtained from early convalescent sera from cases of dengue, Japanese encephalitis and chikungunya infections in man. Fractions from the 19S and 7S zones of the density gradient were pooled, dialyzed against phosphate buffered saline and concentrated by lyophilzation. HI and CF antibody titers of the original serum and the pooled fractions were measured against homologous and heterologous viral antigens. Antigens used were prepared by sucrose-acetone treatment of infected suckling mouse brain.

Results summarized in Table 34 indicate that the 19S pools have HI activity but no CF activity. The 7S pools, on the other hand have both HI and CF activity. The tests done with the Japanese encephalitis and chikungunya antisera indicate that the CF activity of the 7S fraction was specific for the homologous antigen.

The 19S pools were tested by single radial diffusion and immunoelectrophoresis to determine the content of specific immunoglobulins. Results indicated that the 19S pools contained Ig-M in concentrations of 25 to 65 mg% and traces of Ig-G. The 7S pools contained Ig-G and Ig-A with no detectable Ig-M. The HI antibody activity of the 19S pools was entirely 2-mercapto-ethanol sensitive and the HI activity of the 19S pools was entirely 2-mercapto-ethanol sensitive and the HI activity of the 7S pools was resistant to 2-mercapto-ethanol. Therefore, the antibody activity of the 19S pools was due to the Ig-M content.

Experiments were carried out to determine if 19S globulin known to have HI activity, but not CF activity, could block the complement fixing action of whole serum in vitro. Several methods were tried and the most effective was as follows: The CF antigen to be used in the test was incubated with 19S antibody pool (diluted 1:10 or 1:20) for 18 hours at 4°C. Following incubation with the 19S antibody, the antigen was added to the diluted serum in microtiter plates, complement was added, the mixture incubated again for 18 hrs. at 4°C prior to addition of the hemolytic system. The results of these experiments indicate that 19S antibody effectively blocks complement fixation by whole antiserum under the conditions described.

To determine the specificity of this blocking reaction 19S globulin pools from cases of dengue, chikungunya, and Japanese encephalitis were tested for blocking activity against homologous and heterologous antiserum. The results tabulated in Table 35 clearly showed that anti-dengue 19S globulin blocked the CF reaction between dengue antigen, and dengue antiserum, but failed to block the CF reaction between chikungunya antigen and antiserum, or Japanese encephalitis antigen and antiserum. Similarly anti-chikungunya 19S globulin and anti-JE 19S globulin blocked the homologous CF reaciton between antigen and whole antiserum, and failed to block heterologous CF reaction.

The results obtained indicate that Ig-M antibodies bind specifically to the combining sites of CF antigen without fixing complement. The antibody binding is effective in preventing subsequent reaction of complememt fixing antibody with the antigen.

Summary:

Factors effecting the plaque reduction neutralization test for dengue virus antibody were investigated. Several dengue virus strains were found to have a significant loss of infectivity when incubated at 37°C for one hour. Sera from several mammalian species contained non-specific heat labile virus inhibitors at dilutions of 1:10. Statistical evaluation of the plaque reduction neutralization test indicated that it was possible to distinguish 2 fold differences in comparative potency of antisera.

A method for identification and serologic classification of dengue viruses using the plaque reduction test with monkey immune serum is described. Results of typing 40 dengue strains confirmed the usefulness of this method for classification of low passage dengue strains.

A new and highly sensitive method for isolation of dengue viruses by direct and delayed plaque methods in LLC-MK_2 cell culture is described, and evidence is presented which confirms the usefulness of the method.

Studies on experimental dengue infections indicated that Macaca irus and Hylobates lar regularly developed viremia when infected subcutaneously with low passage dengue viruses. Primary dengue infections in Macaca irus results in an early 19S antibody response. Secondary infections however, are characterized by very small amounts of 19S and a marked rise in titer of 7S antibody components.

Fractionation of serum collected from patients with dengue shock syndrome using DEAE-cellulose chromatography indicated that most, if not all, of antibody activity present in the serum during the shock phase is Ig-G. No antibody activity due to Ig-M or Ig-A was detected.

Ig-M antibody present in the early convalescent sera of patients with primary dengue, chikungunya, or JE infections was found to lack complement fixing activity. This Ig-M antibody had HI activity and under experimental conditions was able to block complement fixation by whole antiserum.

Title | Virus Diseases of Americans in Southeast Asia

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Objective: To determine the etiology and study the epidemiology of viral illnesses in US military and civilian personnel stationed in Southeast Asia.

Description: Through liason with dispensaries and hospitals in Thailand specimens for virologic and serologic diagnoses were obtained.

Studies on fevers of unknown origin occurring in US military personnel in South Vietnam are being done through the cooperation of the US Army Medical Research Team (WRAIR) Vietnam and the medical staffs of the 93rd Evacuation Hospital and the 8th Field Hospital. Clinical and epidemiologic data and serum specimens were collected in these hospitals and the serum shipped to SMRL for virus isolation, and serologic testing for arboviruses (HI test) leptospirosis, (hemolytic test) and scrub typhus (FA test).

Progress:

Death due to Hemorrhagic Fever in an American Child.

Previous to this report no fully documented case of dengue shock syndrome had been observed in a caucasian in Thailand. The high incidence of dengue HF in Thai children and the absence of cases in caucasians had led to theories that genetic or nutritional differences caused the apparent difference in susceptibility. Recent evidence indicates that cases of dengue shock syndrome in Thai children are associated with a secondary antibody response to dengue infection. This report presents a fatal case of dengue HF in an American child with an immunologic response indicative of a secondary dengue infection.

A 16 month old male child born in Thailand of American parents had always lived in known dengue endemic areas of Thailand. The patient developed fever on 13 June 1966 and was brought to a medical facility on 16 June because of lethargy. Examination on 16 June revealed a fever (104°F), an erythematous circumoral rash and a palpable liver 2 cm. below the right costal margin. Antipyretics and tetracycline therapy were prescibed. On 17 June he appeared improved, total leucocyte count was 4600/mm^3 and the hematocrit was 37%. On 18 June he was admitted to the hospital because of lethargy, vomiting, and low fluid intake.

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Physical examination on admission revealed slight dehydration and lethargy, pulse 100/min, respirations 40/min, temperature 98.8°F. The lungs were clear and no cardiac abnormalities were noted. The liver was enlarged to 4 cm. below the costal margin. Scattered petechiae were seen on the lower extremities. Neurological examination was negative. Admission leucocyte count was 7,600/mm^3, with 42% polymorphonuclear cells, 56% lymphocytes and 2% monocytes. Hemoglobin concentration was 16 gm%, erythrocyte count was 6.7 million/mm^3.

Intravenous fluid therapy with 5% glucose in normal saline was started and erythromycin and dexamethasone were given. The patient suddenly expired 16 hours after admission.

At autopsy, scattered petechiae were present on the lower extremities. The pleural cavities contained about 200 ml. of serous fluid on each side and the peritoneal cavity contained 300 ml. of serous fluid. The liver extended 4 cm. below the costal margin and patchy capsular hemorrhages were present on the anterior surface. The stomach and intestines contained "coffee ground" fluid. Numerous scattered mucosal petechiae were present in the small and large intestine.

Microscopic examination of the liver revealed patchy hepatic necrosis characterized by hyalinization of hepatic and Kupffer cells. Focal perivascular hemorrhages were seen in sections of gastrointestinal tract. The spleen and lymph nodes showed lymphoid depletion and reticuloendothelial cell hyperplasia. Adrenal cortex revealed focal degeneration, and occasional cytolysis of the cortical cells, particularly of the zona glomerulosa and fasciculata.

No virus was isolated from heart blood obtained at death, 5 days after onset of fever. Antibody titers to the 4 dengue serotypes and Japanese encephalitis virus are listed in Table 36. Neutralizing (N) antibody titers showed broad cross reactivity within the dengue group. Sucrose density gradient ultracentrifugation of the serum (Table 37) revealed the presence of a small amount of 19S, mercaptoethanol sensitive antibody in fraction 4, while the majority of the antibody was in the 7S zone, fractions 6-9, and was mercaptoethanol resistant.

The 3 day febrile period followed by lethargy, increasing hepatomegaly, petechial rash, and hemoconcentration is typical of severe dengue hemorrhagic fever. The findings at autopsy of serous effusions, patchy capsular hemorrhages of the liver, petechiae of gastro-intestinal tract, focal hepatic necrosis, lymphoid depletion with associated reticuloendothelial cell hyperplasia of spleen, and lymphnodes and adrenal cortical damage have been described as characteristic findings after death due to dengue hemorrhagic fever. The clinical, and pathological findings, however, are not pathognomonic.

Neutralizing antibody titers following a first (primary) dengue infection are relatively low titered and do not exhibit extensive cross reactions with the other serotypes. Therefore, the high neutralizing antibody titers to all 4 dengue virus serotypes found in this case are indicative of a second infection with a dengue virus. The HI titer of 1 : 5120 on the 5th day after onset of illness is also evidence of a second infection with a dengue virus. Primary dengue infections have little or no HI antibody this eary in the course of the illness.

The presence of 19S HI antibody establishes a recent dengue infection since 19S antibody persists only 3 to 6 weeks after onset of illness in dengue infections. The very high HI antibody titers in the 7S fractions of the sucrose density gradient on the fifth day after onset of illness are found in secondary dengue infections, but not in primary dengue infections.

Clinical, pathological, and serological findings provide strong evidence that the patient died of dengue hemorrhagic fever associated with a dengue virus infection. This case is of interest since it is the first documented case of severe dengue hemorrhagic fever in a caucasian of European descent, and suggests that factors other than genetics contribute to the apparent insusceptibility previously observed. If the "second infection hypothesis" of the pathogenesis of this disease is correct, the low incidence is due to the relatively small chance of foreigners living in Bangkok of contracting two dengue infections. The greater use of screens, repellants, and insecticides by foreigners, coupled with a limited time of residence in the country may be the most important factors.

FUO studies at the 93rd Evacuation Hospital. Between 1 April 1966 and 31 August 1966, an FUO study was carried out on patients admitted to the medical service of the 93rd Evacuation Hospital, Long Binh, with fever (over 101°F), chills, and headache, a negative malaria smear and in whom a specific diagnosis was not made within the first 24 hours. The results of this study were reported in detail in the Annual Progress Report, of the US Army Medical Research Team WRAIR (Vietnam), for the period ending 31 August 1966. The results of diagnostic studies on the 112 patients studied are summarized in table 38 for the purpose of comparison with data obtained in later studies.

A second study was begun in September 1966. All patients admitted to the medical service of the 93rd Evacuation Hospital between 1 September 66 and 15 February 67 with an unknown or uncertain diagnosis and a fever of 101° or higher on the morning following admission or a temperature elevation to 102° or greater during the first full hospital day were admitted to the study. Patients with malaria were admitted to the study if the diagnosis had not been confirmed by the morning following admission and if they met the established fever criteria.

Acute serum was drawn on all patients admitted to the study but convalescent serum was not obtained from those patients in whom a firm diagnosis was made by other means except in selected cases.

Eighty-six patients without firm diagnoses were lost to the study for administrative or operational reasons. Twenty-five paired samples were lost through breakage, spoilage, and other technical reasons. Five patients were evacuated from the area before convalescent serum could be obtained. The remaining 56 were discharged from the hospital before a second serum sample could be drawn. The clinical condition of the patients and the operational requirement for hospital beds prevented further delay in discharge and operational conditions prevented return of the patients.

Results are summarized in Tables 39, 40, and 41. A diagnosis was made on 69% of patients. Compared to the study done in April-August, dengue decreased markedly, chikungunya disease was not seen at all in the period September through February, and leptospirosis increased from 1% to 9% of cases.

The leptospirosis cases all occurred among troops in combat situations in III Corps area. Cases tended to cluster in units, 4 cases came from a single company with onset only a few days apart. Scrub typhus similiarly occurred in troops on jungle operations and the cases in this study were contracted in III Corps.

FUO Study at the 8th Field Hospital. Sera were collected on 96 patients with clinical diagnosis of FUO at the 8th Field Hospital in October and November 1966. Malaria cases were not included in this study. Serologic studies indicated that of the 96 cases 13 were dengue, 1 chikungunya, 1 Japanese encephalitis, 10 Leptospirosis and 8 scrub typhus. The leptospirosis and scrub typhus cases again occurred among combat troops on operations whereas the dengue infections were contracted in or near Nha Trang.

Distribution of arboviruses in Vietnam. In addition to the studies reported above, dengue was also serologically diagnosed in patients at the 3rd and 17th Field Hospital. If all are combined, a serologic diagnosis of dengue was made on 103 patients between March 1966 and February 1967. Cases occurred through the year with the largest number of cases (28) occurring in May and June corresponding to the peak incidence of hemorrhagic fever in Saigon.

Fourteen strains of dengue virus were isolated from acute phase sera. Of these, seven are dengue-1, two are dengue-2, three are dengue-4 and two are not yet identified. Dengue-1 strains came from cases originating in Saigon, Bien Hoa, Long Binh and Cu Chi. Dengue-2 strains came from Saigon and Ton San Nhut, and the dengue-4 strains came from Ton San Nhut and Bien Hoa. The unidentified strains came from Nha Trang and Duc My.

Chikungunya disease occurred in patients from Saigon, Long Binh, Bien Hoa and Tay Ninh. Only 13 cases were proven, and all occurred during the monsoon season (May-September).

Serologic confirmation of the diagnosis of Japanese encephalitis was obtained in 21 cases of acute central nervous system infections in US personnel. Five cases occurred in Da Nang in August 66, 4 occurred in Saigon or Ton San Nhut, 2 in Long Binh, 1 in Bien Hoa, 1 in Tay Ninh, 1 in Di An, 1 in Quang Duc province, 1 in An Khe, and 3 in Qui Nhon. Japanese encephalitis was also confirmed in 5 Vietnamese children in Quang Ngai where over 100 cases of encephalitis among Vietnamese children are reported annually.

Summary:

A fatal case of hemorrhagic fever in an American child was studied by serologic methods. Infection due to dengue virus and a secondary type antibody response was found.

Studies on fevers of unknown origin occurring in U.S. military personnel in South Vietnam indicated that dengue, leptospirosis, scrub typhus and chikungunya are significant causes of this syndrome. The relative prevalence of these infections varies with the season and with operational factors affecting exposure under combat conditions.

Dengue infections are common throughout III Corps in South Vietnam. In 1966 three dengue serotypes were identified as etiologic agents of FUOs in U.S. military personnel.

Japanese encephalitis was shown to cause central nervous system disease in widely separated regions of South Vietnam.

Title: Ecology of Arboviruses in Thailand

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Objectives:

a. To determine the ecologic factors which affect the maintenance and dissemination of arboviruses causing human disease in Thailand.

b. To develop laboratory techniques for support of field investigations.

Description:

Continued field collections of wild vertebrate sera created an urgent need for a rapid but reliable serological antibody screening technique. This need led to the development of a BHK-21 cell metabolic inhibition test for the detection of Japanese encephalitis (JE), Sindbis and chikungunya (Chik) neutralizing antibodies.

Field studies concerned with the maintenance and transmission of JE virus in and around the Red Cross Horse Farm at Bang Phra have been continuing. The mosquito light trapping program has been expanded, and relative physiologic a , of two vector species, Culex tritaeniorhynchus and C. gelidus, were determined by dissection. At the same time, virus isolation attempts were made from mosquito pools in an attempt to correlate relative abundance and physiologic age with infection. Serological monitoring of a new group of susceptible horses on the farm and of wild vertebrates was conducted in an attempt to determine the time of transmission and to identify suspect reservoir species for further field investigations.

Taxonomic studies designed to give a firm base to the ecological studies have also been continuing. Intensive collections of materials in direct support of the virus studies have been carried out in the Bang Phra area. Additional extensive collections have been made in various areas in Thailand to add to distributional and life history data of Thai birds and mammals and to make the SMRL reference collection more complete.

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Recovery of virus from parous Anopheles mosquitoes collected for malaria field studies have been attempted.

A reported epizootic of equine encephalitis in Nakorn Pathom was investigated.

Progress:

BHK-21 Cell Metabolic Inhibition Test. Extensive serologic surveys require the use of a simple, reproducible and specific laboratory antibody assay system. In our experience, the HI test as a screening device for non-human vertebrate sera is complicated by (1) non-specific reactions despite acetone or kaolin extraction of sera (2) removal of specific antiviral macroblogulin by kaolin treatment and (3) misleading intragroup cross-reactions. A neutralization test having the simplicity of the HI test was needed for serologic surveys. The metabolic inhibition test experiments conducted in our laboratory represent an attempt to develop such a neutralization test for three of the arboviruses present in Thailand.

The BHK-21 cell metabolic inhibition (MI) test is similar to MI tests with other cell-virus systems reported by others. An acid (yellow color) indicates cell survival and an alkaline pH (red or purple color) represents virus-induced cell destruction BHK-21 MI tests employing both microtiter and large disposable plastic plates have been used with local strains of Sindbis, Japanese encephalitis (JE), and chikungunya viruses.

Studies with the BKM-21 cell macro (large disposable plate) and microtiter metabolic inhibition test were carried out. A series of experiments were done manipulating several of the variable factors to achieve consistent results and clear color contrast. Experiments with various cell concentrations indicated that 4,000 cells in 0.1 ml growth medium per well for the microplates and 25,000 in 0.5 ml per well for the large plate was optimal. A variety of factors in pH control were examined, including CO_2 vs aerobic incubation, heavy vs extra heavy mineral oil at several volumes per well, mineral oil vs Saran wrap covering of plates, and several concentrations of sodium bicarbonate. Some of the early inconsistencies in cell growth were traced to inadequately cleaned plates. Our present procedure calls for washing plates (0.7% haemosol) followed by thorough rinsing (at least 8 times) in tap water and demineralized water. The plates are then sterilized by ultraviolet light.

The volumes per well of test reagents used are as follows:

Reagents	Microtiter plate	Large plate
Heavy mineral oil	0.08 ml	1.5 ml
Virus (in M 199 + 20% FBS) (100 TCD_{50})	0.025 ml	0.1 ml
Serum dilutions in M199+ 20% FBS	0.025 ml	0.05 ml
Cells (live count) in M199+ 5% FBS	4,000	25,000
Volume of cell suspension	0.1 ml	0.5 ml
$NaHCO_3$ in medium	0.0007%	0.0015%
Over-feeding with 19S+ 6% FBS	0.5 ml day 4	0.8 ml day 5

The procedure used is as follows:

(1) Serum dilutions are added to the plates.
(2) The virus dose is added (100 TD LD_{50}) next.
(3) Serum-virus mixtures are incubated at 37°C for 2 hours.
(4) Cell suspensions are added.
(5) Oil is added (may be done first in micro plates).
(6) Extra heavy mineral oil is applied to top outside edges of plate.
(7) Plates are covered with sterile Saran wrap (oil from step 6 should seal Saran wrap to plate).
(8) Plates are incubated at 37°C in aerobic incubator.
(9) Saran wrap is removed and cells in each well overfed (do not replace Saran wrap after feeding).
(10) Plates are reincubated for 2-3 days and read.

Addition of Saran wrap has produced very sharp end-points and virtually eliminated troublesome mycotic contamination.

Comparative microtiter, macro plate and tube neutralization tests with JE virus indicate correlative results by these methods when acute and convalescent phase sera from clinical JEV encephalitis cases were tested (Table 42). Failure of several convalescent phase sera to neutralize in the first test was due to virus "break-through" probably owing to low avidity of the early sera and high sensitivity of the BHK-21 cells to JE virus. Retesting of several of these sera increasing the serum virus incubation period from one to two hours and, in the case of the microtiter test, lowering the virus dose, significantly increased serum titers (Table 42). Similar tests were carried out with Sindbis and chikungunya viruses and reference antisera in microtiter, macro plate and tube neutralization tests. The Sindbis test was carried out before the discovery that longer (two-hour) virus-serum incubation was necessary. Even with the shorter incubation time, the microtiter method was as sensitive as the tube method with two of the three sera tested, whereas the macroplate method appeared to be less sensitive than either (Table 43). The chikungunya tests were carried out with the two hour serum-virus incubation. In this case virus "break through" and rapid cell to cell spread in the established tube monolayers resulted in significantly lower serum neutralizing titers in tubes than in either macro or micro plates, where addition of a cell suspension to the serum virus-mixture did not permit this to happen (Table 43). The microtiter method was the most sensitive of the three techniques compared.

Experiments to determine intragroup neutralization specificity have begun. In two trials there was no cross neutralization between chikungunya and Sindbis viruses. Three group B antisera, Japanese encephalitis hyperimmune rabbit. Tembusu hyperimmune mouse, and dengue 2 immune monkey sera were tested against JE (Nakayama) virus. There was no heterologous neutralization by the sera, which had homologous plaque reduction neutralization titers of 1:900, 1:1280 or>, and 1:1500, respectively. When serum titers from cases of dengue fever and viral encephalitis were compared by hemagglutination-inhibition (HI) and microtiter MI tests, the magnitude of the acute phase-convalescent phase changes were in complete agreement for the encephalitis cases (Table 44). The one encephalitis case with a four-fold group B HI titer rise also had a four-fold MI neutralization rise, doubtless diagnostic of JE encephalitis. In four other encephalitis cases where HI titers were high and relatively fixed and JE encephalitis was suspected, the MI neutralization test failed to show four-fold rises of acute to convalescent phases titers. In these cases either the MI test was not adequately sensitive or these HI titers represent old group B infections not associated with the encephalitis. Perhaps most significant was the failure to demonstrate four-fold acute-convalescent phase cross reacting antibody titer rises to JE virus by MI tests in three cases of secondary dengue infections (Table 44). In these infections a four-fold or greater HI antibody rise occurred to both dengue and JE viruses, with all convalescent phase serum titering to over 1:5,000. These results suggest that it may be possible to exclude JE, serologically, as an etiologic agent where a broadly cross-reactive group-B HI response occurs following infection with other group B viruses.

The BHK-21 cell micro MI test has proved to be reproduceable, reasonably accurate, easy to perform, comparatively inexpensive and sparing of field-collected sera which are often in very limited quantity. The limited sensitivity is of less concern where one wishes to exclude all false positive even if a few true positives are also excluded. Studies on the ecology of Japanese encephalitis virus. Studies on the ecology of Japanese encephalitis virus at Bang Phra, an area of year round virus activity, have been continuing. Field activities have focused on two main problems: (1) The capture of large numbers of Culex tritaeniorhynchus and C. gelidus to assess virus infection, relative abundance and physiologic age and (2) the capture of birds and mammals to identify which species were present on the area and to determine which of these species might be involved in virus maintenance.

Bang Phra Mosquito Study. Between February 1966 and February 1967, mosquitoes were obtained by the Department of Medical Entomology from 655 light trap collections made in four areas in the vicinity of the horse farm operated by the Red Cross Society of Thailand at Bang Phra in Cholburi province. The most abundant species captured in the light traps were: Culex fuscocephalus, C. gelidus, C. tritaeniorhynchus, Aedes lineatopennis, A. mediolineatus, A. vexans, Anopheles aconitus, Mansonia annulifera and M. uniformis. A total of 181, 284 mosquitoes belonging to the above species were tested for the presence of virus, and viral isolates were obtained from pools of C. gelidus, C. tritaeniorhynchus, A. lineatopennis, A. mediolineatus and A. vexans. The peaks in abundance of all five species occurred during the rainy season (May-October), While the populations of C. gelidus, C. tritaeniorhynchus, A. vexans and A. lineatopennis were observed to reach two peaks, during the months May-June and again in September-October, the A. mediolineatus population demonstrated a single peak during July and August during which the populations of the other four species were exhibiting an unexplained decline (Figure 12). The aquatic stages of the two Culex species favor semi-permanent frequently polluted bodies of water such as slow-moving streams, paddy-fields and ponds, while the three Aedes species characteristically breed in temporary rain-pools. All five species are known to feed upon large domestic animals, and the greatest numbers were collected at Bang Phra during this period from two light traps located in the vicinity of a cattle barn and a horse stable, respectively. That those mosquitoes were in fact feeding on livestock at Bang Phra was borne out by the results of agar-gel diffusion tests run against the gut contents of engorged mosquitoes from these light trap collections. Details of the agar-gel tests are given under the section on mosquito studies.

The age composition of C. gelidus and C. tritaeniorhynchus populations at Bang Phra were studied during this period through dissection and examination of ovaries of mosquitoes from light trap collections. Parous were distinguished from nulliparous females by the presence of absence of terminal coils on the ovarian tracheoles. A significant rise in the proportion of older (parous) C. gelidus females occurred in March and again in June; it was during those two months that the only isolations of viral agents were obtained from C. gelidus at Bang Phra during this period (Table 45). Unfortunately, the numbers of C. tritaeniohynchus dissected during this same period were too small to indicate whether the population of that species exhibited similar changes in age composition. Interestingly, all of 12 JE antibody-free horses resident at the Bang Phra horse farm developed JE antibodies between 2 June and 2 July, the time when many parous C. gelidus females were present and when both C. gelidus and C. tritaeniorhynchus were particularly abundant.

Additional mosquito pools were submitted by Bang Phra Red Cross personnel to the Virology Department for recovery of viruses. The total numbers of mosquitoes tested for the presence of virus is given in table 46.

Wild Mammals and Bird Sampling. Through the last 12-month period, birds and mammals were live-trapped in the Bang Phra area. All specimens were bled for serological testing, birds being bled from the external juglar vein and mammals by cardiac puncture or by rupture of the retroorbital sinus. One half of the specimens were sacrified for the museum skin collection and for virus isolation attempts from organ (spleen, brain and kidney) suspensions. The other animals were marked and released at the point of

capture. Many of these animals were frequently recaptured, and served as field sentinels of virus activity. Sera are being tested for neutralizing activity to JE, chikungunya and Sindbis virus in the metabolic inhibition test. Approximately 0.1 ml. of blood drawn in the field was inoculated into each of two I oz. bottles containing LLC-MK_2 cell monolayers. These cells were returned to the laboratory where they were checked for CPE and then overlaid with agar to determine the presence of plaque-forming agents. To date all virus isolation attempts from vertebrates have been negative.

Serological tests have been conducted with over 600 sera representing about 40 species, approximately one third of the total number of specimens collected. Of these, low percentages of sera from 13 species have neutralized JF virus, four species have neutralizing antibody to sindbis virus and two species have neutralizing antibody to chikungunya virus (Table 47). A comparatively small percentage of the total individuals in any reacting species had neutralizing antibody. Sera from five specimens neutralized JE virus at the end of the rainy season (October-November) and were negative when bled and tested two weeks to two months later. This could be accounted for on several bases: (1) the positive MI test reactions may have been spurious (2) two of the second sera had to be diluted 1 : 10 and all second sera were tested against 300 $TCLD_{50}$ whereas first sera were tested against 70 $TCLD_{50}$, all of which could make the second test less sensitive than the first, and (3) antibody levels may have rapidly declined between the first and second bleedings, and the MI test was not sensitive enough to detect antibody in diluted, low-titered serum. No. MI test negative to positive conversions have been found, although many of the sera from serially bled individuals remain to be tested. In no case did a single sera react with all viruses, and only one sera reacted with two viruses, suggesting that these sera did not contain substances capable of non-specificitically inactivating arboviruses in general.

Results of the MI tests suggest infection of rodents, small resident birds and possibly bats in the Bang Phra area. The ecological significance of these observation is unknown. The association of neutralizing properties of the MI test positive sera with their immune globulins has yet to be demonstrated. Tests to these ends are underway.

Isolation of new viruses. During the past year, viral agents were recovered and reisolated from Bang Phra mosquito species which had previously not been associated with viruses in Thailand. Two viruses were recovered from Aedes mediolineatus, two from Ae. lineatopennis and one from Ae. vexans (Table 48). Identification of four of these has been attempted. Two of these, BKM-367/66 from Ae. mediolineatus and BKM-589/66 from Ae. lineatopennis appear to be group B Arboviruses (Table 49) somewhat similar to but not identical with Tembusu, JE and dengue 4 viruses (Table 50) but identical with each other and with BKM-448/66 (Tables 51,52). BKM-457/66, from Ae. vexans, failed to react by HI test with either arbovirus A or B grouping serum.

Taxonomy of Thailand Vertebrates. Work on the taxonomy of birds and mammals continues to progress. A field handbook on the identification of rats of Thailand was prepared and published. The main collecting effort has been at Bang Phra, in support of the Japanese encephalitis virus studies being done there.

Additional areas ecologically disimilar to the central plains area have also been sampled, particularly a broad-leaved evergreen subtropical forest area along highway 23 between Korat and Kabinburi. In this area Suncus etruscus, Rattus sladeni and an apparently new species (perhaps genus) of yellow horseshoe bat were collected. In a nearby area, Nakorn Nayok, a melenistic variety of Rattus raja was found. In the northewest mountains of Thailand, in the Mae Sariang area, Rattus nitidus was collected by SMRL personnel for the first time.

In the central plains area, Mus musculus was found inhabiting a grain warehouse in Thonburi, and a bird mist netting program on Koh Kret was carried out in an area where pervious capturing and releasing of birds had resulted in banding of many individuals.

Attempted virus recovery from Anophelines. Many anopheline species commonly bite man. Their possible role as arbovirus vectors in Thailand is unknown. As a by-product of malaria studies done by the Department of Medical Entomology, salivary glands from parous (hence, having had one or more previous blood meal) female *Anopheles* mosquitoes of 19 species were pooled by time, place and species and tested for presence of virus by inoculation into suckling mice and LLC-MK_2 cell cultures. To date, all attempts at virus recovery have been unsuccessful, but the numbers of mosquitoes tested has been very small, (1, 147 individual mosquitoes in 219 pools).

Equine Encephalitis in Nakorn Pathom Caused by Japanese Encephalitis Virus. In Nakorn Pathom, Thailand, from August through November, the Department of Livestock Development (Thai Government) estimated 30 equine deaths due to encephalitis among a total population in the area of about 300 horses. Only the last case was seen by SMRL Virology and Veterinary Medicine personnel, who along with Dept. of Livestock Development personnel, obtained acute and convalescent phase sera from that animal. At that time sera were collected from 29 horses and tested for the presence of Japanese Encephalitis (JE) virus antibody. All sera were tested by the micro hemagglutination-inhibition (HI)., complement-fixation (CF) and metabolic inhibition (MI) neutralization tests. Results of these tests (Table 53) demonstrate that horses in the Nakorn Pathom area are infected with JE or a closely related virus. The presence of CF antibody suggests fairly recent infection. In one case it was possible to associate JE infectian with clinical encephalitis, serologically (Table 54).

Summary:

A reliable BHK 21 cell micro metabolic inhibition test was developed for detecting JE, sindbis and chikungunya antibodies. This test is at least as sensitive as the tube neutralization test. The test appears to be quite specific, and requires very small amounts of reagents.

A large number of mosquitoes were collected in light traps at Bang Phra. All five of the most commonly collected species, *Culex gelidus, C. tritaeniorhynchus, C. fuscocephalus, Aedes vexans, Ae. mediolineatus* and *Ae. lineatopennis,* were most abundant during the rainy season. *C. gelidus* the only species for which enough dissections permitted evaluation, had significant rises in the proportion of older (parous) females in March and again in June. It was during these times that viruses were recovered from this species. Between 2 June and 2 July 1966 all of 12 JE antiboby-free horses at Bang Phra developed antibody.

During the 1966-1967 collecting period, twelve viral agents were recovered from Bang Phra mosquitoes, nine by us and three by Red Cross personnel. Identification of these viruses has not been complete, but at least three are JE virus. Five of the viruses recovered from Aedes species are unlike any of the arboviruses previously known to be present in Thailand. Three are group B agents and appear to be identical. One is neither group A nor group B and one has not yet been tested for group reaction.

Twenty-nine individuals of 13 vertebrate species from Bang Phra have reacted with JE virus in the metabolic inhibition test. A number adequate for calculation of antibody prevalence rates has not yet been tested, but these data suggest wild vertebrate infection in the area.

Taxonomic studies have resulted in the publication of a field identification book of Thai rats. One new rat and bat species has been added to the SMRL reference collection. The bat may represent a previously unknown species.

Attempts at recovery of viruses from parous *Anopheles* mosquitoes have been unsuccessful.

About 30 equine cases of encephalitis occurred in Nakorn Pathom in August-November 1966. The last case, the only one seen by us, was confirmed as JE on serologic grounds. This represents the first

proved case of JE in horses in Thailand, although others have been suspected. Of 29 horses, 28 had JE antibody. Of these, two horses had signs of neurological damage, suggesting that wide spread JE infection among horses in this area had occurred.

Publications

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2. Halstead, S.B., Yamarat, C., Russell, P.K. Hemorrhagic fever in Thailand and South Vietnam; newer knowledge regarding etiology. Jap. J. of Exp. Med. and Biol., in press.

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6. Bhamarapravati, N., Boonyaratvej, S., Russell, P.K. Encephalitis and pneumonitis due to chikungunya virus: report of a fatal case. J. Med. Assoc. Thailand, 49:627, 1966.

7. Russell, P.K., Chumdermpadetsuk, S., Piyaratn, P. A fatal case of dengue hemorrhagic fever in an American child. Pediatrics, in press.

8. Russell, P.K., Nisalak, A., Sukhavachana, P., Vivona, S. A plaque reduction test for dengue neutralizing antibodies. J. Immunol., in press.

9. Russell, P.K., Nisalak, A. Dengue virus identification by plaque reduction neutralization test. J. Immunol., in press.

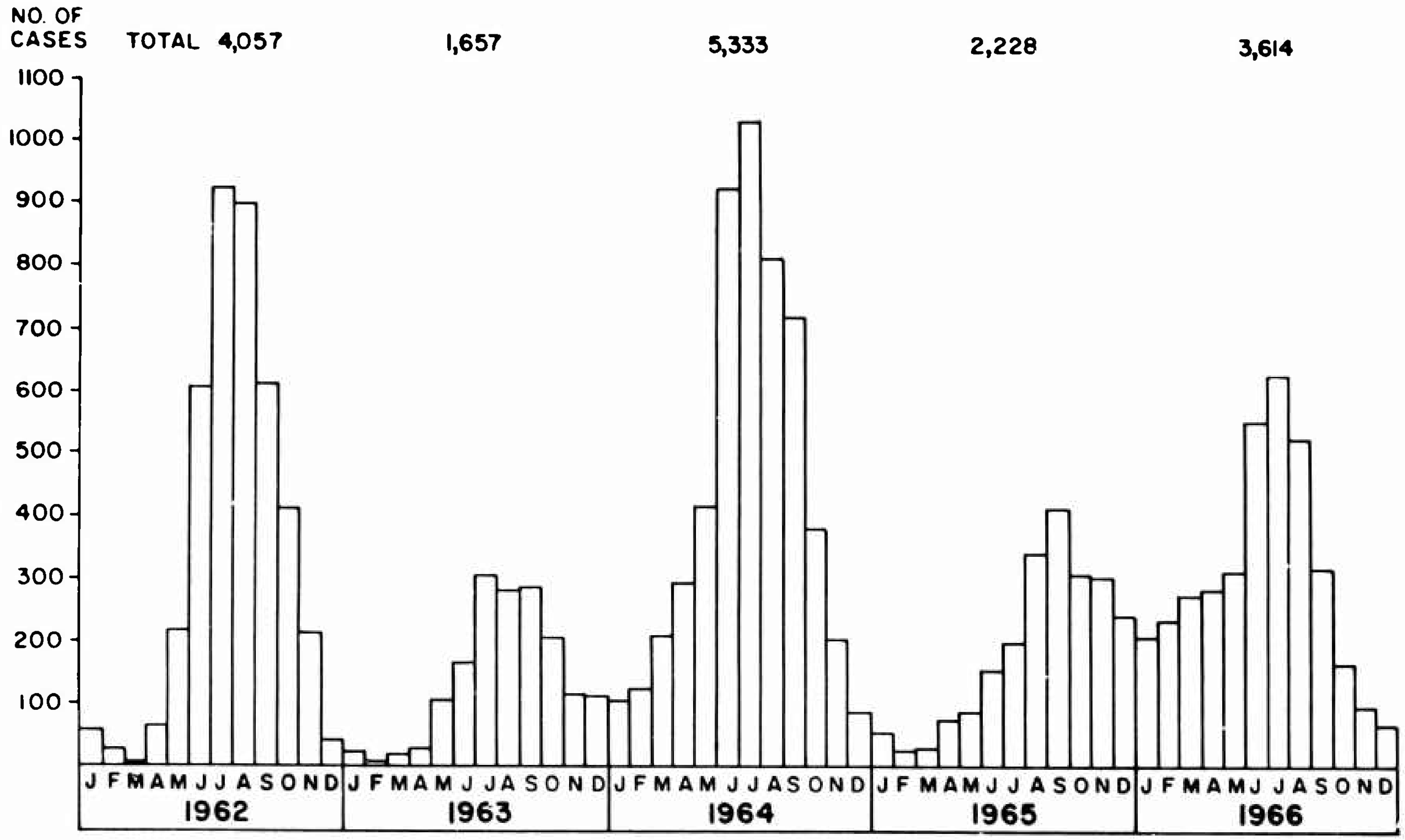

FIG. I CASES OF THAI HEMORRHAGIC FEVER ADMITTED TO BANGKOK AND THONBURI HOSPITALS, 1962—1966.

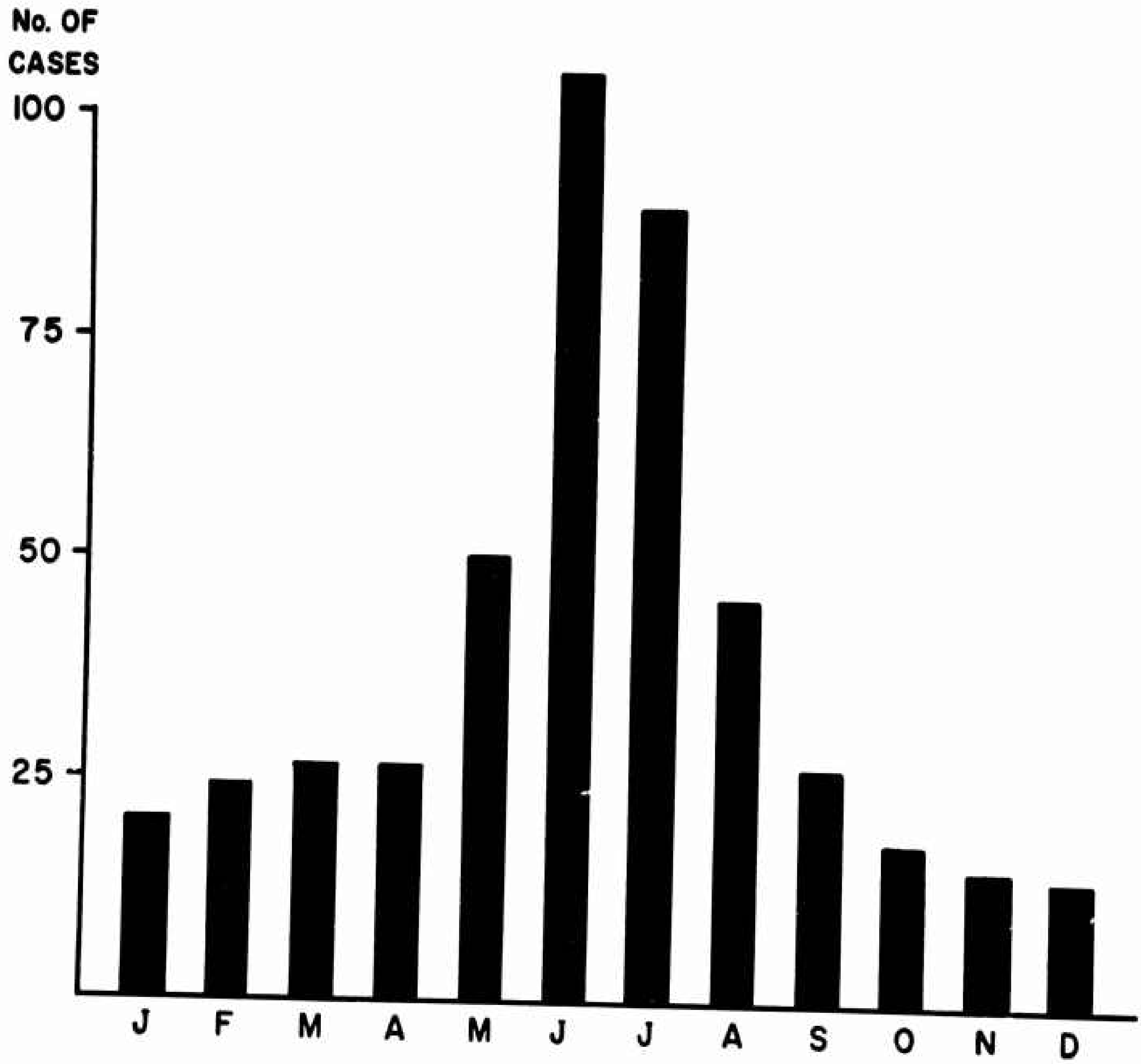

FIG. 2 CASES OF HEMORRHAGIC FEVER ADMITTED TO 3 SAIGON HOSPITALS DURING 1966 BY MONTH.

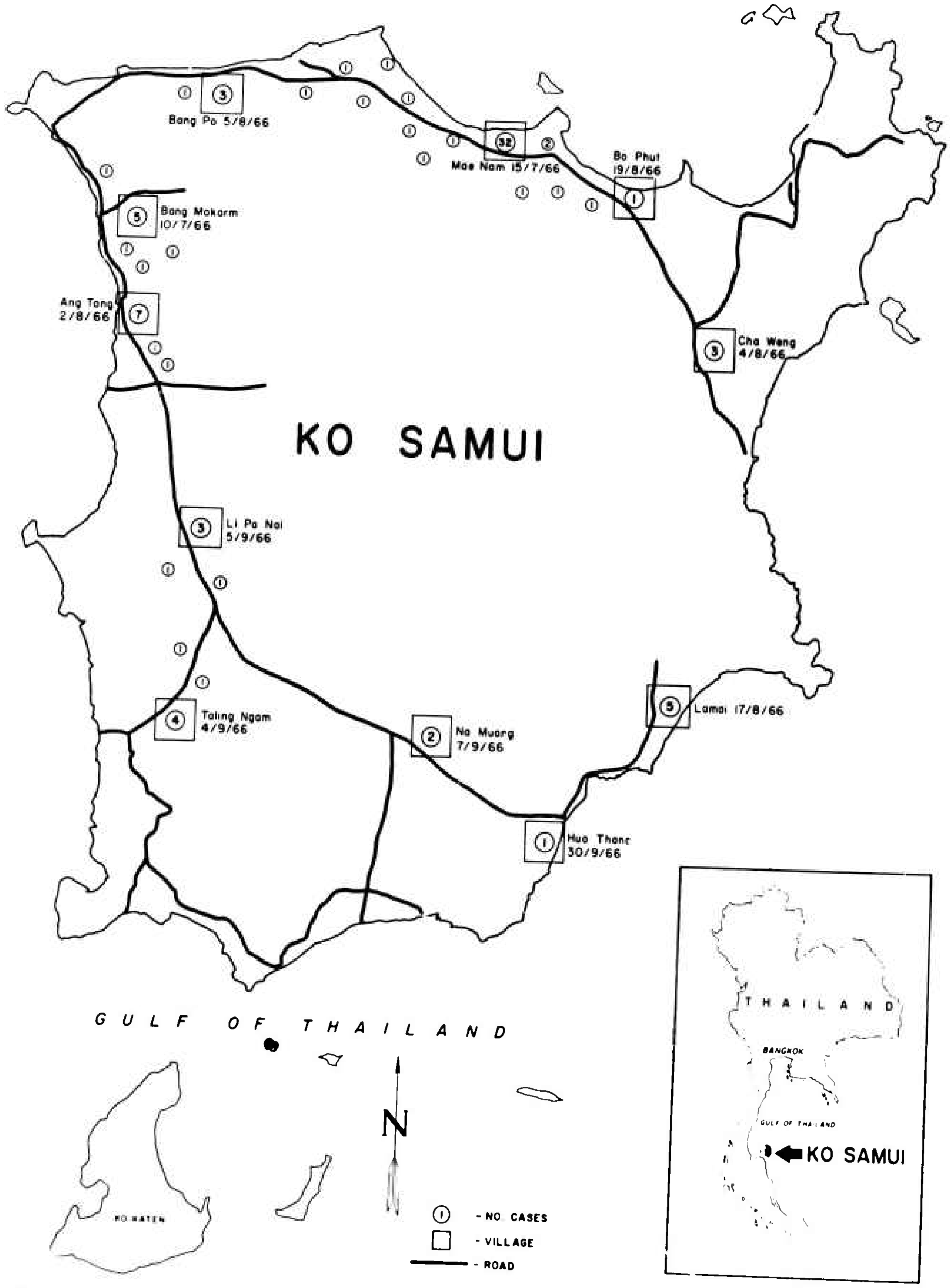

Figure 3. Map of Koh Samui showing location of cases and date of initial case in each village.

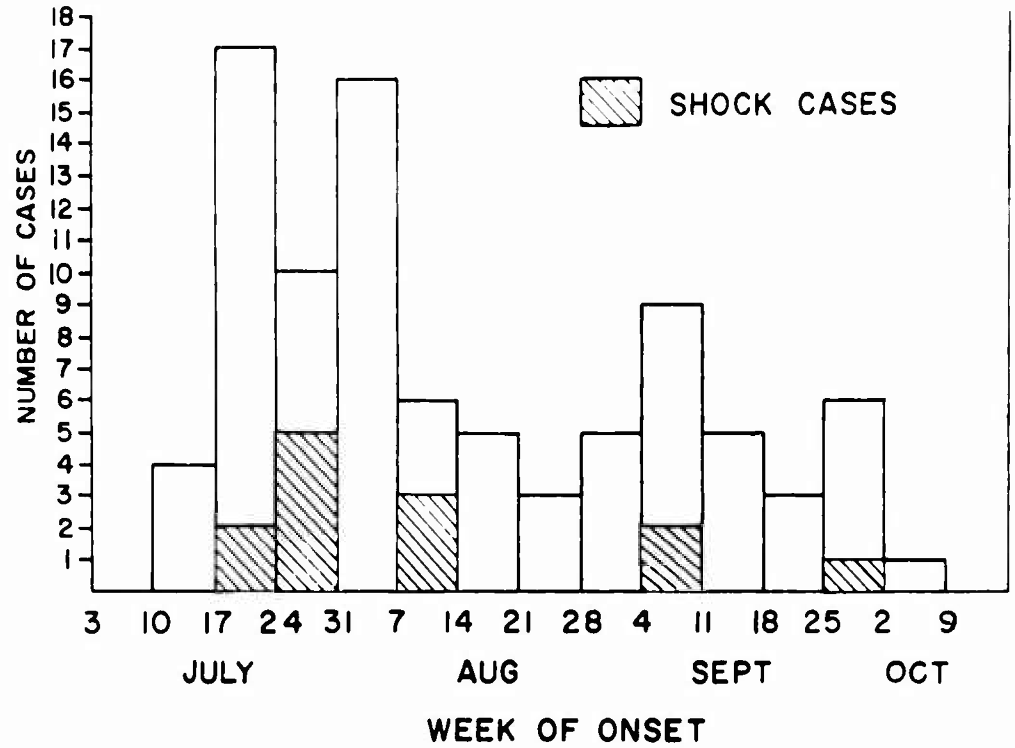

Figure 4. Distribution of 90 dengue cases by week of onset

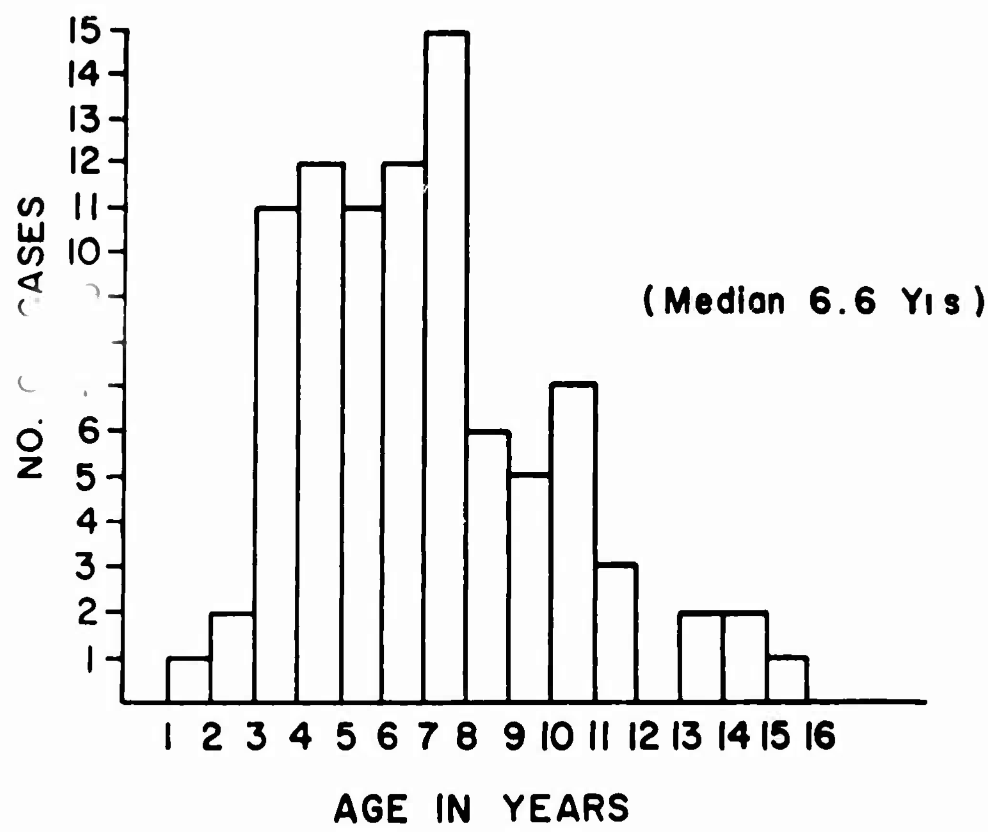

Figure 5. Age distribution of 90 dengue cases

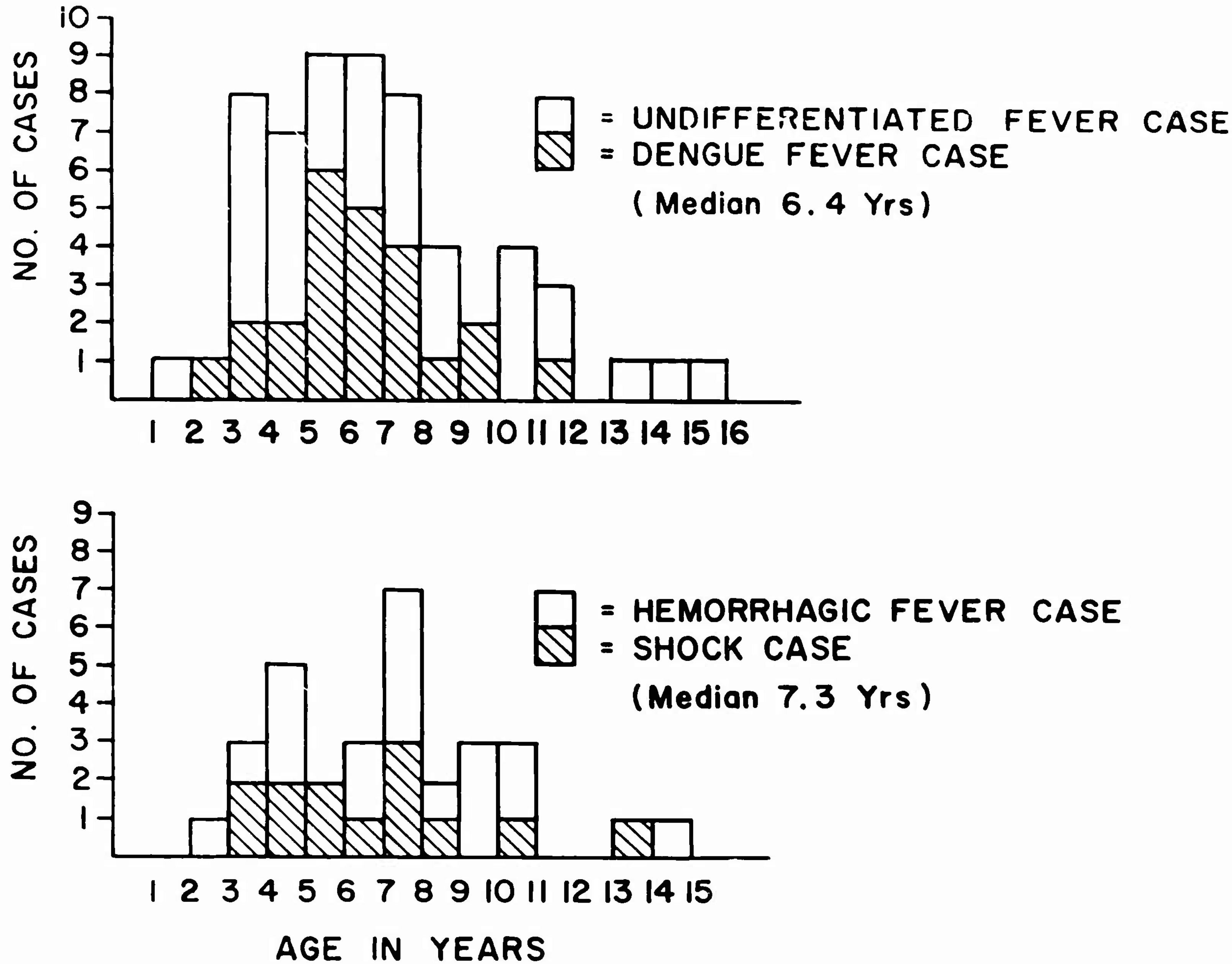

Figure 6. Age distribution of 90 dengue cases by clinical syndrome

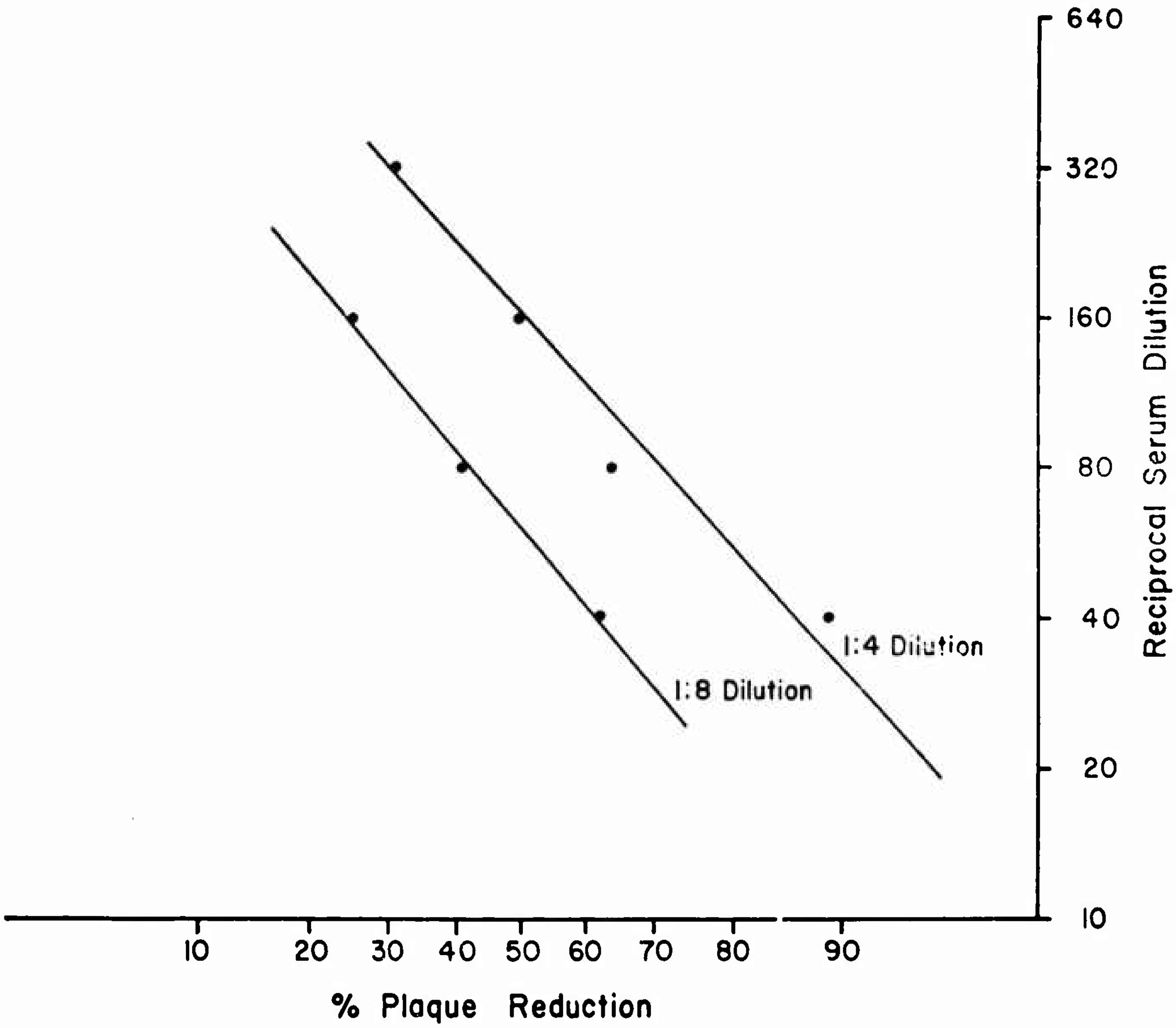

Figure 7. Plaque reduction neutralization of dengue—I (Hawaii) virus by 2 dilutions of dengue—I (Hawaii) antiserum

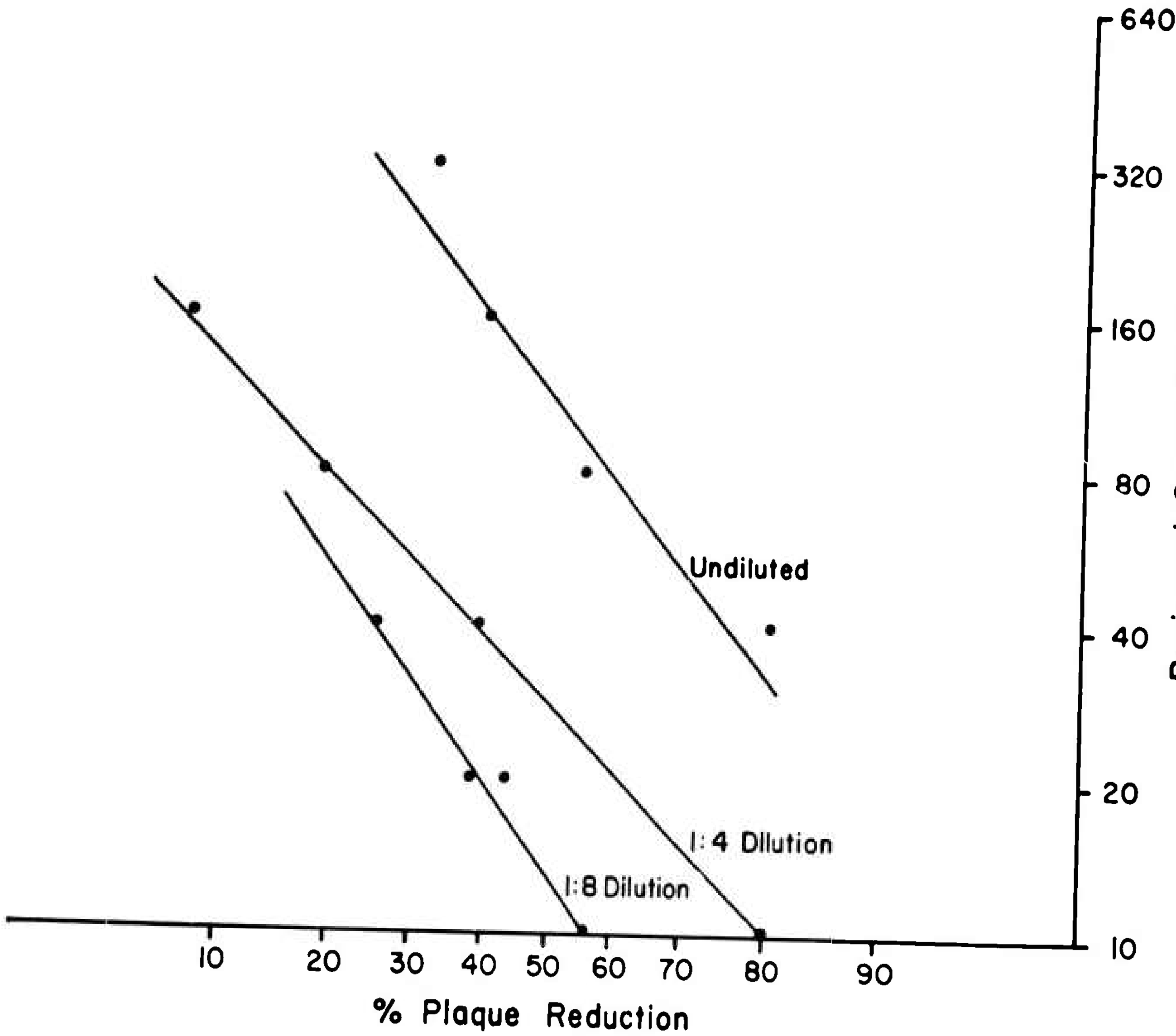

Figure 8. Plaque reduction neutralization of TH—Sman virus by 3 dilutions of TH—Sman antiserum.

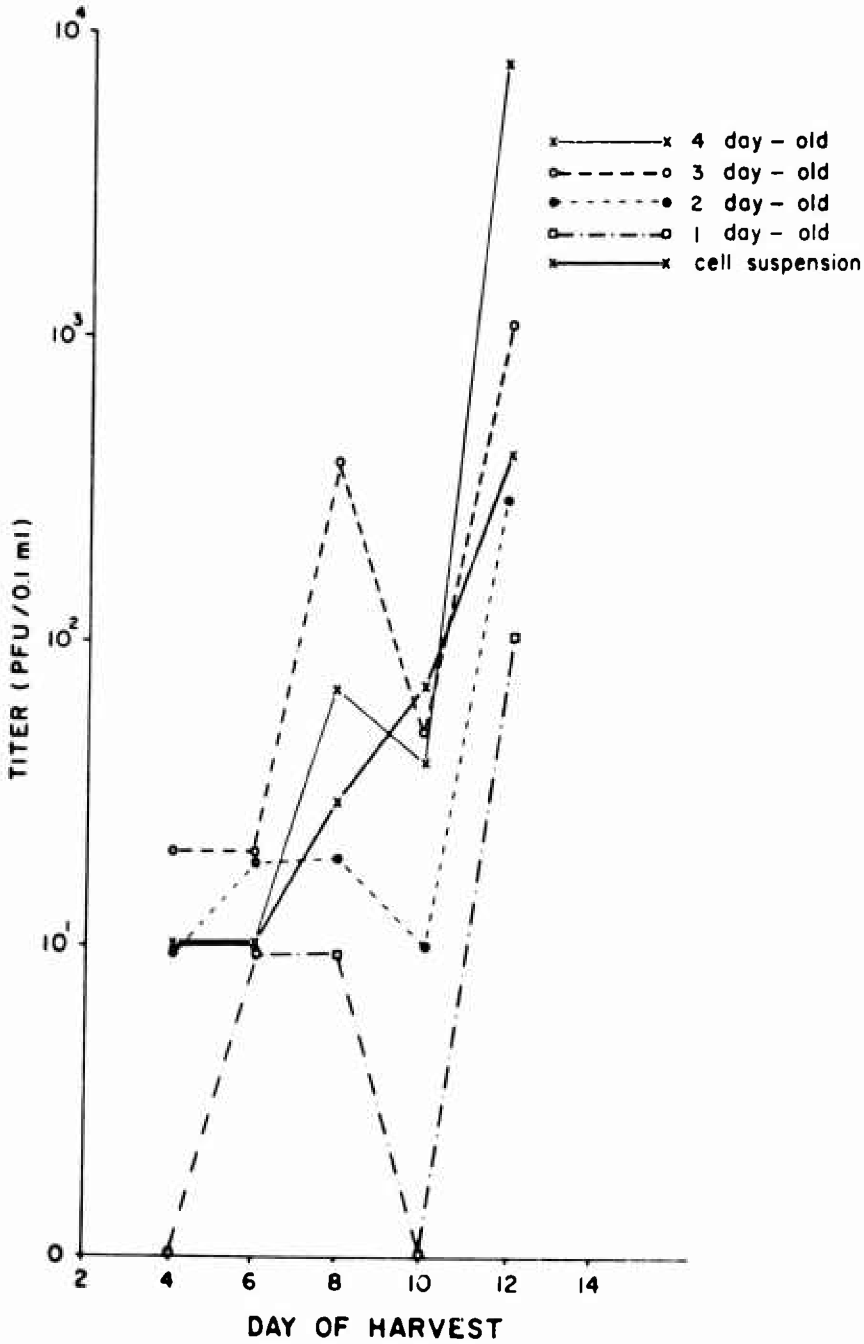

Figure 9. Growth of dengue-2 (BKM-540) virus in LLC-MK$_2$ cells of various ages

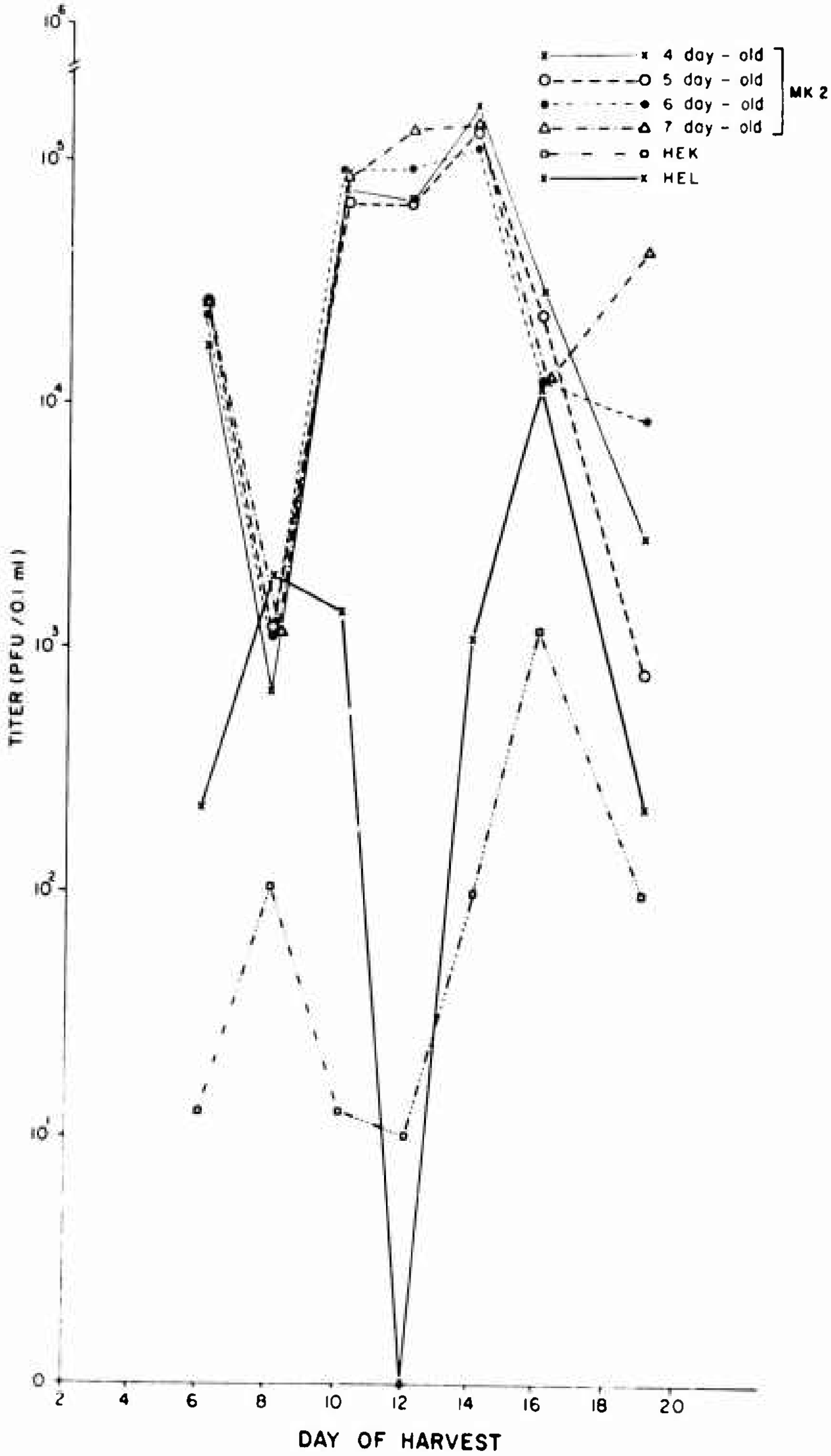

Figure 10. Growth of dengue–2 (BKM–540) virus in LLC–MK_2 cells of various ages and in human embryonic lung and human embryonic kidney cells.

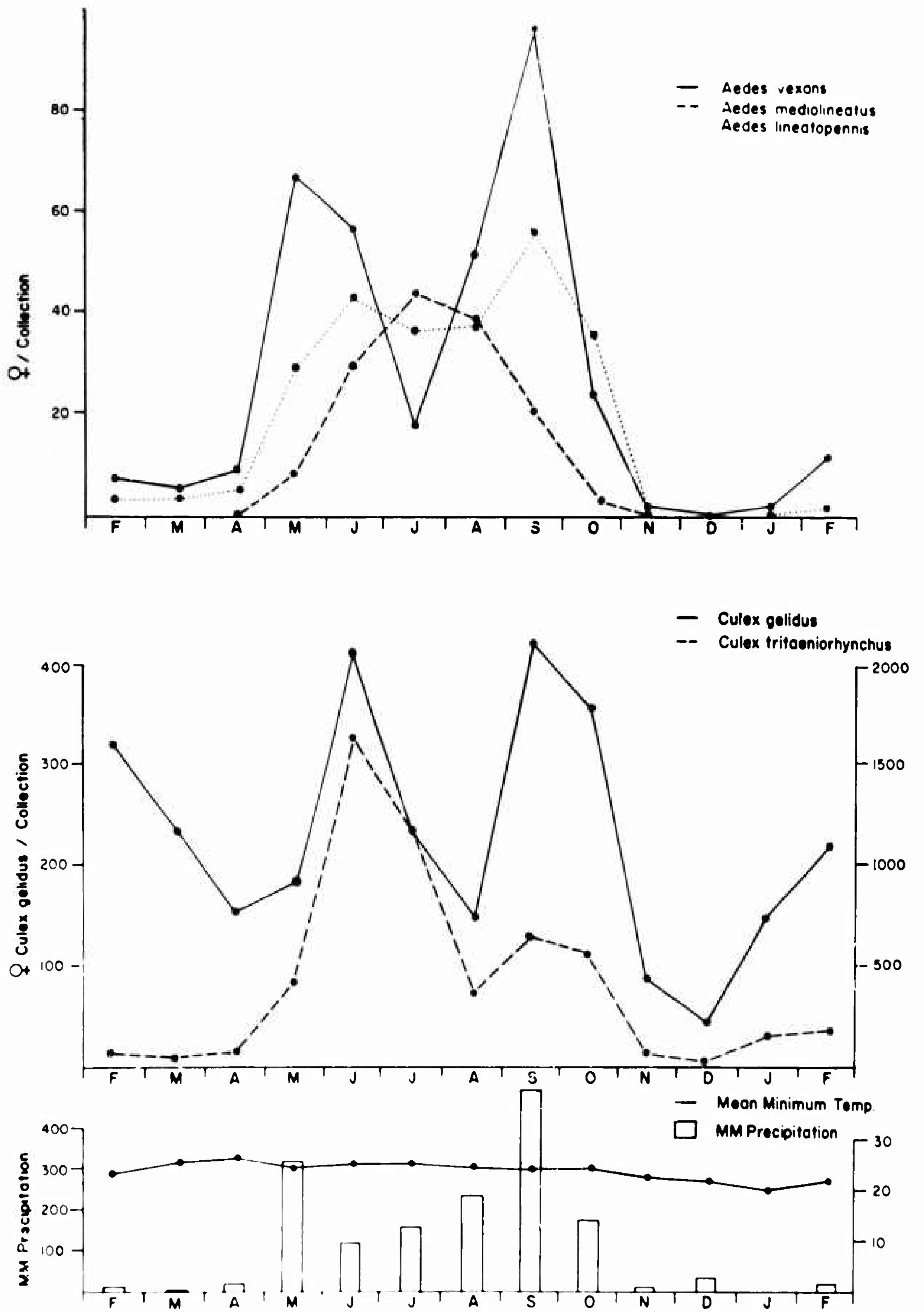

Figure 11. Light trap collections of Culex gelidus, Culex tritaeniorhynchus, Aedes lineatopennis, Aedes mediolineatus and Aedes vexans at Bang Phra, 1966–67.

Table 1. Age specific hospitalization rates for hemorrhagic fever, Bangkok–Thonburi, 1965–1966.

Age (yr)	1965		1966	
	Cases	Rate/1000*	Cases	Rate/1000*
<1	246	2.9	330	3.9
1	197	2.4	190	2.3
2	260	3.3	275	3.5
3	280	3.7	352	4.6
4	291	3.9	404	5.5
5	222	3.1	431	6.1
6	191	2.9	408	6.2
7	156	2.4	282	4.4
8	115	1.9	260	4.3
9	85	1.6	190	3.5
10	65	1.3	180	3.5
11	57	1.2	149	3.0
12	46	1.0	109	2.4
13	13	0.31	51	1.2
14	13	0.34	30	0.78
15	4	0.003	22	0.018

*based on 1960 census

Table 2. Age distribution of 452 hospitalized cases of hemorrhagic fever in Saigon 1966.

Age (yr)	Cases		Deaths	
	No.	%	No.	%
<1	14	3.1	3	[illegible]
1	27	6.0	5	[illegible]
2	38	8.4	2	[illegible]
3	97	21.6	12	16.9
4	117	25.9	25	35.2
5	49	10.8	10	14.1
6	38	8.4	7	9.9
7	21	4.6	3	4.2
8	15	3.2	2	2.8
9	5	1.1	0	
10	10	2.2	0	
11	8	1.8	0	
12	5	1.1	2	2.8
>12	8	1.8	0	

Table 3. Distribution of cases by clinical syndrome and etiology.

Syndrome	Etiology		
	Dengue	Unknown	Total
Shock	13	0	13
HF	18	8	26
DF	24	12	36
UF	35	29	64
Totals	90	49	139

Table 4. Distribution of 90 dengue cases by clinical syndrome and serologic class.

Syndrome	Serologic Response		
	Primary	Secondary	Pos., N.E.C.*
Shock	0	9	4
HF	1	9	8
DF	3	18	3
UF	5	25	5
Totals	9	61	20

* Positive, not exactly classifiable (see text)

Table 5. Distribution of dengue Cases by age and sex.

Age (yr)	Male	Female	Total
<1	0	0	0
1	1	0	1
2	1	1	2
3	6	5	11
4	6	6	12
5	2	9	11
6	6	6	12
7	6	9	15
8	2	4	6
9	2	3	5
10	3	4	7
11	1	2	3
12	0	0	0
13	0	2	2
14	1	1	2
15	1	0	1
Total	38	52	90

Table 6a. Dengue viruses from human cases by dengue type and clinical syndrome.

Clinical Syndrome (No. cases)	Numbers of Strains Isolated			
	Type 1	Type 2	Type 3	Untyped
Shock (13)	0	1	1	1
HF (26)	0	0	2	0
DF (36)	1	1	3	2
UF (64)	0	6	0	3
Totals	1	8	6	6

Table 6b. Distribution of 15 dengue virus strains by serotype, area and date of recovery.

Village	Virus Type	No. of Strains	Date (s) of Recovery
Mae Nam	1 2 3	1 5 1	20 July 20 26 July; 2,8,22 Aug. 31 July
Taling Ngam	2 3	1 2	11 Aug. 4 Aug. 6 Sept.
Ang Tong	3	3	31, Aug: 6, 29 Sept.
Li Pa Noi	2	1	10 Sept.
La Mai	2	1	5 Sept.

Table 7a. Number of dwellings found infested with Aedes aegypti in three Koh Samui villages, 1966–1967

Month	Mae Nam	Ang Thong	Taling Ngam
July, 1966	13/13*	25/25	–
August, 1966	14/15	–	–
February, 1967	76/120	76/76	14/30

* (No infested / no checked)

Table 7b. Sources of A. aegypti and A. albopictus larvae collected on Koh Samui, November 1966.

Species	Source (Number of Collections) Artificial Containers*	Natural Containers**
Aedes aegypti	10	3
Aedes albopictus	17	42
Both species	8	3

* Artificial containers: water jugs, cans drums, etc.
**Natural containers: coconut shells, husks and bracts.

Table 7c. Summary of collections of adult A. aegypti and A. albopictus from Koh Samui, November 1966.

Species	Indoors: Number Times Collected	Indoors: Number Mosquitoes Collected	Outdoors: Number Times Collected	Outdoors: Number Mosquitoes Collected
Aedes aegypti	25	86	1	2
Aedes albopictus	12	47	9	72

Table 8. Effect of heat on dengue virus strains.

	Time (minutes)	Viruses							
		Hawaii (D–1)	TH-Sman (D–1)	#10572 (D–1)	N. G. "C" (D–2)	TH-36 (D–2)	#10286 (D–2)	H-87 (D–3)	H-241 (D–4)
		%	%	%	%	%	%	%	%
37°C	15	69*	88	84	82	88	98	98	88
	30	66	80	71	—	75	97	98	98
	60	46	72	52	64	28	64	88	74
	90	41	67	42	—	28	—	80	68
42°C	15	8	80	49	65	62	90	70	61
	30	0	60	25	—	20	88	60	48
	60	0	44	0	38	0	48	44	18
	90	0	12	—	—	0	—	20	10

*Percent virus survival compared to zero time control, determined by plaque assay

Table 9. Dengue plaque reduction activity of antibody free, fresh, unfrozen sera.

		Virus			
Sera		D-1	D-2	D-3	D-4
Human-A	undil	73*	90	95	65
	1:5	10	18	25	12
	1:10	0	10	0	0
Human-B	1:5	11	20	0	30
	1:10	5	15	0	0
Monkey V90	undil	67	—	69	—
	1:5	21	28	0	28
	1:10	17	30	0	26
Monkey V92	1:5	39	0	9	23
	1:10	0	0	0	0
Rabbit	1:5	—	40	20	0
	1:10	24	16	24	0
Guinea pig	1:5	30	30	38	7
	1:10	19	20	0	0
Mouse	1:10	—	65	—	—

*% reduction compared to controls

Table 10. Prototype and newly isolated dengue virus strains studies.

Dengue Virus Strain	Host* and Passage	Origin	Year Isolated	Associated Disease**	Dengue Serotype
Dengue–1 (Hawaii)	sm–124	Hawaii	1944		1
Dengue–2 (N.G. "C")	sm–26	New Guinea	1944		2
Dengue–3 (H–87)	sm–21	Philippines	1956		3
Dengue–4 (H–241)	sm–24	"	1956		4
TH–36	sm–16	Thailand	1958		2
TH–Sman	sm–13	"	1958		1
2383–62	sm–4	Thailand	1962	U	3
2557–62	sm–5	"	"	D	3
5987–62	sm–6	"	"	HF	3
648–63	sm–7	"	1963	HF	3
2403–63	tc–1, sm–6	"	"	HF	4
2443–63	sm–7	"	"	HF	4
10044	sm–3	"	1964	HF	2
10286	sm–4	"	"	HF	2
10572	sm–7	"	"	HF	1

Table 10. (Cont'd)

Dengue Virus Strain	Host* and Passage	Origin	Year Isolated	Associated Disease**	Dengue Serotype
11340	sm – 7	Thailand	1964	HF	2
12900	sm – 3	,,	,,	D	1
13507	sm – 6	,,	,,	D	1
13819	sm – 4	,,	,,	D	1
14486	sm – 6	,,	,,	U	4
14580	tc – 3	,,	,,	D	1
14670	sm – 4	,,	,,	U	3
14962	sm – 3	,,	,,	HF	3
16005	sm – 4	,,	,,	HF	3
16572	sm – 4	Philippines	,,	HF	4
16603	sm – 5	Philippines	,,	HF	4
16681	tc – 3	Thailand	,,	HF	2
16727	tc – 3, sm – 3	,,	,,	HF	4
16876	sm – 6	,,	,,	HF	3
17048	sm – 3	,,	,,	HF	3
18280	sm – 7	,,	,,	HF	1
18320	tc – 2, sm – 7	,,	,,	D	3
Pak 18	tc – 3	Pakistan	,,	D	3
T502066	sm – ?, tc – 3	Tahiti	,,	D	3

Table 10. (Cont'd)

Dengue Virus Strain	Host* and Passage	Origin	Year Isolated	Associated Disease**	Dengue Serotype
20731	sm-10	Thailand	1965	HF	2
20833	sm-7	"	"	HF	2
21153	sm-3	"	"	HF	3
21868	tc-3	"	"	HF	2
22448	sm-2	"	1966		1
23751	tc-3	Vietnam	"	D	4
23753	sm-3	"	"	D	2
24001	tc-3	"	"	D	1
24007	tc-3	"	"	D	1
24038	tc-1	"	"	D	4
24177	tc-3	"	"	D	1
24705	tc-2	Thailand	"	HF	3
24960	tc-1	"	"	HF	3

* sm-Suckling mice, tc-Tissue culture

** D-Dengue fever, HF-Hemorrhagic fever, U-Undifferentiated febrile illness.

Table 11. Homologous and heterologous neutralizing antibody titers of monkey antisera to prototype strains of dengue virus.

Viruses	Antisera					
	D-1	D-2	D-3	D-4	TH-36	TH-Sman
Dengue-1	300*	0	90	10	0	100
Dengue-2	0	1400	30	13	1700	0
Dengue-3	0	0	350	0	0	0
Dengue-4	0	0	14	150	0	0
TH-36	0	2500	NT**	NT	2500	NT
TH-Sman	70	0	NT	NT	0	115

* Reciprocal of 50% plaque reduction titer, 0 = <10

** NT = not tested

Table 12. Neutralizing antibody titers of monkey antisera to dengue viruses related to type 1.

Viruses	Antisera									
	D.1	D.2	D.3	D.4	TH-36	TH-Sman	12900	14580	18280	22448
Dengue-1	300*	0	90	10	0	100	520	100	260	420
TH-Sman	70	0	NT	NT	0	115	530	280	> 640	> 640
10572	66	0	25	0	0	80				
12900	50	0	40	0	0	120	670		380	450
13507	35	0	0	0	0	50	410		530	580
13819	25	0	0	0	0	90				
14580	105	20	35	20	15	150		> 640		
18280	40	0	0	0	0	70	420		425	> 640
22448	110	0	15	0	0	140	400		480	490
24001	100	0	40	0	0	160				
24007	160	12	30	0	0	120				
24177	180	0	20	0	10	170				

* Reciprocal of 50% plaque reduction titer 0 <10

Table 13. Neutralizing antibody titers of reference antisera against dengue viruses related to type 2.

Viruses	Antisera					
	D–1	D–2	D–3	D–4	TH–36	TH–Sman
Dengue–2	0*	1400	30	13	1700	0
TH–36	0	2500	NT	NT	> 2560	
10044	0	> 640	60	20		
10286	0	600	50	10		
11340	0	350	10	0	190	0
16681	0	500	80	0		
20721	0	> 640	40	0	> 640	0
20833	0	> 320	10	0	> 320	0
21868	0	240	10	0	100	0
23753	0	270	15	0		

*Reciprocal of 50% plaque reduction titer; 0 <10

Table 14. Neutralizing antibody titers of reference antisera to dengue viruses related to type 3.

Viruses	Antisera							
	D 1	D 2	D 3	D 4	TH 36	TH Sman	14670	Pak 18
Dengue 3	0*	0	350	0	0	0	100	40
2383 62	0	0	350	0	0	0		
2557 62	0	0	130	0	0	0		
5987 62	0	0	120	0	0	0		
648 63	0	0	150	0	0	0		
4670	0	0	200	0	0	0	130	
4962	0	0	160	0	0	0		
16005	0	0	180	0	0	0		
Pak 18	0	0	270	0	0	0		55
16876	0	0	190	0	0	0		
17048	0	0	130	0	0	0		
18302	0	0	300	0	0	0		
21153	0	0	200	0	0	0		
24705	0	0	300	0	0	0		
T 502066	0	0	70	0	0	0		

* Reciprocal of 50% plaque reduction titer; 0 = <10

Table 15. Neutralizing antibody titers of dengue-4 antisera to reference virus strains.

Viruses	Antisera		
	Dengue-4 Monkey	Dengue-4 Mouse	No. 14486 Monkey
dengue-1	10*	10	0
dengue-2	15	30	0
dengue-3	0	20	0
dengue-4	150	> 320	130
TH-36	10	10	0
TH-Sman	0	0	0

* Reciprocal of 50% plaque reduction titer; 0 <10

Table 16. Neutralizing antibody titers of reference antisera to dengue viruses related to type 4.

Viruses	Antisera						
	D 1	D 2	D 3	D 4	TH 36	TH Sman	No. 14486
Dengue 4	0*	0	0	> 320	0	0	130
2403 63	0	0	0	140	0	0	270
2443 63	0	0	0	100	0	0	
14486	0	0	0	110	0	0	> 320
16572	0	0	0	100	0	0	100
16603	0	0	0	280	0	0	200
16727	0	0	0	80	0	0	160
23751	0	0	0	> 320	0	0	
24038	0	0	0	> 320	0	0	

*Reciprocal of 50% plaque reduction titer; 0 = <10

Table 17. Identification of dengue viruses of isolate from patients on Koh Samui.

Virus	Passage Level	Antisera D.1	D.2	D.3	D.4	Virus Type
24366	tc 3	80*	0	0	0	1
24367	tc 4	0	50	0	0	2
24453	tc 3	0	40	0	0	2
24464	tc 4	0	25	0	0	2
24546	tc 2	0	0	160	0	3
24552	tc 4	0	160	0	0	2
24705	tc 2	0	0	300	0	3
24718	tc 5	0	160	10	0	2
24742	tc 4	0	25	0	0	2
24801	tc 4	0	300	0	0	2
24956	tc 2	0	0	80	0	3
24967	tc 3	0	100	0	15	2
24969	tc 1	0	0	140	0	3
24970	tc 1	0	0	80	0	3
25076	tc 3	0	40	0	0	2
25163	tc 2	0	0	230	0	3
Homologous viruses		400	450	160	130	

*Reciprocal of 50+ plaque reduction titer, 0 <10

Table 18. Identification of dengue viruses isolated from mosquitoes from Koh Samui.

Virus	Mosquito Species	Passage Level	Antisera D 1	D 2	D 3	D 4
BKM 536 66	Ae. aegypti	sm 4	0*	450	20	0
BKM 540 66	,, ,,	tc 3	0	50	0	0
BKM 542 66	,, ,,	tc 3	0	100	0	0
BKM 547 66	,, ,,	sm 3	0	120	40	0
BKM 551 66	,, ,,	sm 4	0	1000	40	0
,, ,, ,,	,, ,,	tc 4	0	40	0	0
BKM 773 66	Ae, albo	tc 4	0	30	0	0
BKM 875 66	,, ,,	tc 2	0	40	0	0

*Reciprocal of 50+ plaque reduction titer, 0 <10

Table 19. Results of plaque reduction neutralization tests with monkey antisera against tissue culture and suckling mouse strains of dengue-2 viruses.

	Monkey Antisera			
Viruses	dengue-2 (N.G. "C")	dengue-2 (TH-36)	BKM-551 tc-4	BKM-551 sm-4
Dengue-2 (New Guinea "C" sm-26)	450*	450	65	1000
Dengue-2 (TH-36, sm-16)	160	320	25	160
BKM551, tc-4	40	30	20	40
BKM551, sm-4	2000	NT**	60	160

* Reciprocal of 50% plaque reduction titer

** NT Not tested

Table 20. Comparison of dengue virus isolation from human serum and blooded mosquitoes by direct and delayed plaque isolation techniques.

Dengue Type	Direct Only	Delayed Only	Both Methods
1	0*	0	1
2	0	4	8
3	0	1	5
Untyped	0	5	9
Totals	0	10	23

* No. of strains isolated

Table 21. Number of passages required for suckling mouse mortality by 15 dengue agents in acute phase human serum.

	No. of Strains Causing Mortality at Indicated Passage		
Dengue Type	1st	2nd	3rd
1	1	0	0
2	0	2	4
3	1	1	2
Untyped	1	0	3
Totals	3	3	9

Table 22. Virus isolations from mosquitoes from Koh Samui.

Pool No.	Species	Plaques in LLCMK-2 cells		Suckling Mice		Virus type
		direct	delayed	ill	CVR	
BKM-497	_Ae. aegypti_	3	nt*	0	12/12	?
M-527	,, ,,	3	nt	0	12/12	?
BKM-536	,, ,,	5	nt	11/12**	-	dengue-2
BKM-540	,, ,,	0	nt	0	7/12	dengue-2
BKM-542	,, ,,	20	nt	0	11/12	dengue-2
BKM-545	,, ,,	8	nt	0	0	?
BKM-546	,, ,,	30 +	nt	2	8/10	?
BKM-547	,, ,,	30 +	nt	6/16	nt	dengue-2
BKM-551	,, ,,	30 +	nt	4/16	nt	dengue-2
BKM-773	_Ae. albo._	0	31	0	11/12	dengue-2
BKM-842	,, ,,	0	35	0	0	?
BKM-844	,, ,,	18	nt	0	12/12	?
BKM-875	,, ,,	12	nt	0	10/12	dengue-2

* nt not tested

** No. ill or dead/No. inoculated

*** CVR-resistent to 100 LD_{50} dengue-2 challenge

Table 23. Viremia in monkeys (Macaca irus) following subcutaneous injection of dengue viruses.

Dengue Type	Virus strain and Passage	Inoculum (pfu)	Days Viremia Detected
1	14580 BS C 1 p 4	$10^{[illegible]}$	7,8,9,10
1	18280 BS C 1 p 4	$10^{[illegible]}$	6,7,8,9,10
1	22448 BS C 1 p 4	$10^{[illegible]}$	7,8,9
1	12000 BS C 1 p 4	$10^{[illegible]}$	4,5,6,7,8,9,10
1	Hawaii sm 123	10^4	7,8,9,10
1	TH Sman sm 14	$10^{[illegible]}$	5,6,7,8
1	13507 sm 8	$10^{[illegible]}$	7,8,9,10
3	AP 16 BS C 1 p 3	$10^{[illegible]}$	7
4	11220 BS C 1 p 4	$10^{[illegible]}$	4,5,6,7,8
4	14486 BS C 1 p 3	$10^{[illegible]}$	5,6
1	12900 BS C 1 p 4	$10^{[illegible]}$	1,2,3,4,5,6,7,8,9
4	2443 63 sm 4 LLCMK2 p 2	$10^{[illegible]}$	5,7

Table 24. Viremia in gibbons (Hylobates lar) following subcutaneous injection with dengue 2 virus*.

Gibbon No.	Days Viremia Detected	Maximum Titer (pfu/ml.)
S 21	4,5,6,7,8	4.5x10[illegible]
S 29	6,7,8,9	2.5x10[illegible]
S 30	3,4,5,6	1.5x10[illegible]
S 34	3,4,5,6,7	2.5x10[illegible]
S 38	2,3,4,5	4.1x10[illegible]
S 41**	6	15
S 42**	6,7,8	30

* 100 pfu of No. 10044 (3rd BS C 1 passage)

**S 41 and S 42 had a dengue 1 infection 5 months previously

Table 25. Appearance of 19S and 7S dengue HI antibodies following a primary dengue 2 infection and a secondary dengue 1 infection one year later.

Day After Inoculation	Monkey A 21				Monkey A 37			
	1st Infection*		2nd Infection**		1st Infection*		2nd Infection**	
	19S	7S	19S	7S	19S	7S	19S	7S
2	0***	0	0	160	0	0	0	80
4	0	0	0	160	0	0	0	80
6	0	0	0	160	0	0	0	80
8	0	0	0	160	0	0	0	80
10	10	0	0	80	80	0	0	80
12	80	20	0	640	320	40	0	320
14	160	80	0	1280	320	80	0	1280
21	80	160	0	1280	160	320	0	1280
28	80	160			160	640	0	1280
35	20	320			80	320		
42	0	160			0	320		
60	0	320			0	320		

* Dengue 2 virus, 120 pfu

** Dengue 1 virus, 1000 pfu

***Reciprocal HI titer in highest titer fraction in 19S zone or 7S zone, 0 <10

Table 26. Specificity of 19S and 7S dengue neutralizing antibody 14 days after dengue 2 infection.

Antibody Fraction		Prototype Dengue Viruses					
		D 1	D 2	D 3	D 4	TH 36	TH Sman
Monkey A 21	19S	0*	45	0	0	30	0
	7S	0	180	0	0	100	0
Monkey A 39	19S	10	15	0	0	30	0
	7S	20	1,200	90	0	1,800	30

* Reciprocal of 50% plaque reduction titer

Table 27. Neutralizing antibody response to dengue 1 infection one year following dengue 2 infection.

Monkey No.	Days After Inoculation	Viruses D 1	D 2	D 3	D 4
Monkey A 21	0	15*	1800	30	2
	21	> 2560	> 2560	45	200
Monkey A 37	0	0	750	0	0
	21	> 2560	> 2560	320	300

* reciprocal of 50% plaque reduction titer

Table 28. Homologous neutralizing antibody titers following dengue 1 infection one year after dengue 2 infection.

	Days after Inoculation 2	4	6	8	10	14
Monkey A 21	40*	60	40	40	200	> 2560
Monkey A 37	20	20	60	40	40	> 2560

* Reciprocal of 50% plaque reduction titer

Table 29. Density gradient centrifugation of serum from a primary dengue infection*

Day of Illness	Fraction No. 1	2	3	4	5	6	7	8	9	10
5	0**	0	0	0	0	0	0	0	0	0
6	0	0	20	20	0	0	0	0	0	0
7	0	0	20	20	0	0	0	0	0	0
8	0	0	20	40	0	0	0	0	0	0
10	0	20	40	80	10	20	80	0	0	0
19	0	0	40	20	0	80	160	20	0	0
30	0	0	0	20	0	40	80	40	0	0

* Case No. HFI 735, [illegible] hemorrhagic fever due to dengue 3 virus.
**Reciprocal of HI titer, 0 10

Table 30. Density gradient centrifugation of serum from a secondary dengue infection*

day of Illness	Fraction No. 1	2	3	4	5	6	7	8	9	10
4	0**	0	0	0	0	40	160	20	0	0
6	0	0	0	0	20	320	1280	160	40	0
8	0	0	20	0	80	640	1280	320	80	40
19	0	0	0	0	40	320	1280	160	40	0
31	0	0	0	0	20	640	1280	160	40	40

*Case No HFI 720, dengue shock syndrome due to dengue 2 virus.
**Reciprocal of HI titer, 0 10

Table 31. Dengue HI titers of sera and serum pools from patients with dengue shock syndrome

Case No.	Serum No.	Day After Onset*	HI Antibody Titer D.1	D.2	D.3	D.4
HFI-773	25327	6**	320	640	2560	2560
	25328	7**	640	2560	> 20480	2560
	25335	8	2560	2560	> 20460	> 20480
	25338	9	2560	2560	> 20480	> 20480
	pool		1280	1280	> 20480	> 20480
HFI-747	23548	3**	5120	10240	> 20480	> 20480
	23565	7	> 20480	> 20480	> 20480	> 20480
	23567	9	> 20480	> 20480	> 20480	> 20480
	23617	13	> 20480	> 20480	> 20480	> 20480
	pool		> 20480	> 20480	> 20480	> 20480
HFI-749	23735	5**	> 20480	> 20480	> 20480	> 20480
	23745	10	> 20480	10240	> 20480	> 20480
	23818	20	> 20430	> 20480	> 20480	> 20480
	23843	30	1280	1280	1280	5120
	pool		10240	10240	10240	20480
HFI-744	22516	5**	640	640	5120	640
	22575	6	5120	5120	20480	2560
	22581	8	2560	1280	20480	1280
	22629	20	1280	1280	20480	2560
	pool		5120	1280	20480	2560

* Day after onset of illness
** Days on which shock was observed

Table 31. (cont'd)

Case No.	Serum No.	Day After Onset*	HI Antibody Titer D-1	HI Antibody Titer D-2	HI Antibody Titer D-3	HI Antibody Titer D-4
HFI-737	22277	5**	320	640	1280	640
	22307	10	> 20480	> 20480	> 20480	> 20480
	22324	20	10240	10240	> 20480	5120
	22361	30	10240	10240	> 20480	5120
	pool		> 20480	> 20480	> 20480	5120
HFI-782	25857	4**	5120	1280	5120	2560
	25858	5	10240	1280	10240	10240
	25878	6	> 20480	5120	> 20480	> 20480
	pool		> 20480	5120	10240	10240

* Day after onset of illness

** Days on which shock was observed

Table 32. Relationship of immunoglobulin concentration to HI antibody titers in fractions from DEAE-cellulose Chromatography of serum pools from patients with dengue shock syndrome.

Case No.	Fraction No.	Immunoglobulin Conc. Ig G	Ig A	Ig M	HI titer*
HFI 773	I	560**	0	0	2560
	II	15	20	0	40
	III	40	0	98	160
HFI 747	I	440	0	0	10240
	II	30	66	0	160
	III	30	tr.***	86	160
HFI 749	I	520	0	0	10240
	II	20	70	0	80
	III	20	tr.***	110	80
HFI 744	I	280	0	0	10240
	II	tr.***	30	0	40
	III	tr.***	0	46	20
HFI 737	I	450	0	0	10240
	II	tr.***	32	0	20
	III	tr.***	0	30	> 20
HFI 782	I	280	0	0	5120
	II	tr.***	31	0	80
	III	tr.***	0	15	40

*Reciprocal of titer vs 8 units of the dengue antigen which gave highest titer.
**Mg.
***Trace

Table 33. Sucrose density gradient ultracentrifugation of serum pool from case HFI 773

	Fraction No. 1	2	3	4	5	6	7	8	9	10
Untreated	0*	0	0	0	80	320	1280	640	40	40
2 ME Treated	0	0	0	0	80	320	1280	640	40	40

*Reciprocal of HI titer vs dengue 3 antigen, 0 <20

Table 34. HI and CF titers of whole sera and 19S and 7S fractions of early convalescent sera from arbovirus infections.

Convalescent Sera		HI Titers** Dengue 2	HI Titers** Chik	HI Titers** JEV	CF Titers*** Dengue 2	CF Titers*** Chik	CF Titers*** JEv
24043 (D)*	whole serum	20480	0	5120	128		
	19S	640			0		
	7S	5120			32		
22241 (D)	whole serum	160	0	80	8		
	19S	20			0		
	7S	80			4		
22631 (D)	whole serum	1280	0	640	16	0	16
	19S	320	0	320	0	0	0
	7S	1280	0	1280	16	0	32
25321 (C)	whole serum	0	320	0	0	8	0
	19S	0	320	0	0	0	0
	7S	0	320	0	0	8	0
25524 (JE)	whole serum	0	0	320	0	0	8
	19S	0	0	320	0	0	0
	7S	0	0	320	0	0	8

*(D) Dengue infection, (C) Chikungunya infection, (JE) Japanese encephalitis infection

**Reciprocal of titer vs 8 units of indicated antigen, 0 <20

***Reciprocal of titers vs 4 units of indicated antigen, 0 <4

Table 35 Inhibition by specific 19S antibody of complement fixation by whole antisera and arbovirus antigens

Type of 19S Antibody Tested for Inhibition	Antidengue Serum vs Dengue Antigen	Aeti-Chik Serum vs Chik. Antigen	Anti-JE Serum vs JE Antigen
None (control)	32	8	8
Anti-degue*	0	8	8
Anti-Chik**	32	0	8
Anti-JE***	32	8	0

* 19S fraction of serum 22631

** ,, ,, ,, ,, 25321

*** ,, ,, ,, ,, 25524

Table 36. Serum antibody titers at time of death (5th day of disease).

Viruses	Neutralizing Antibody Titers*	Hemagglutination-Inhibiting Antibody Titers**
Dengue-1	1:350	1:2560
Dengue-2	1:640	1:5120
Dengue-3	1:600	
Dengue-4	1:240	
Jap. Enceph.	<1:10	1:5120

* 50% plaque reduction titers

** Vs. 8 units of indicated antigen

Table 37. Dengue hemagglutination-inhibition titers* of sucrose density gradient fractions of serum at time of death.

Fraction No.	1	2	3	4	5	6	7	8	9	10
Untreated	0	0	0	40	0	40	320	160	20	0
Mercaptoethanol Treated	0	0	0	0	0	40	320	160	20	0

* Reciprocal of titer vs. 8 units of dengue-2 antigen, 0 <10

Table 38. Results of diagnostic studies on 112 patients with FUO at 93rd Evacuation Hospital, 1 April 66-31 August 66.

Diagnosis	No. of Cases	
Malaria	10	(incl. 2 scrub typhus)
Leptospirosis	1	
Scrub Typhus	11	(incl. 2 malaria)
Dengue	31	
Chikungunya	10	
Other Diagnosis	7	
Total With Diagnosis	68	(60%)
Undiagnosed	44	(40%)

Table 39. Results of diagnostic studies on patients with FUO at 93rd Evacuation Hospital, 1 Sept '6 to 15 Feb 67.

Total patients admitted to study	295
Withdrawn (administrative & operational necessity)	86
Total Studied	209

Final Diagnosis on 209 patients	No. cases	
Malaria	53	(incl 2 scrub typhus)
Leptospirosis	18	
Scrub Typhus	13	(incl 2 malaria)
Dengue	10	
Japanese Encephalitis	5	
Other Diagnosis	46	
Total With Diagnosis	143	(69%)
Undiagnosed	66	(31%)

Table 40. Clinical diagnoses on 46 patients listed above as "other diagnosis".

Mumps	1	Acute bronchitis	1
Pyelonephritis	1	Meliodosis	1
Prostatitis	6	Inf. mononucleosis	6
Lymphogranuloma	1	Serum sickness	3
Strept. pharyngitis	5	Hemolytic disease	1
Amoebiasis	8	Cellulitis/abcess	4
Pneumonia	8		

Table 41. Distribution of cases by month and diagnosis, 93rd Evac. Hosp. FUO Study.

Month	No. Cases	Malaria	Lepto	Scrub	Dengue	JE	Other	Undx
Sept	33	12	4	2	2	1	7	5
Oct	36	8	2	2	1	3	8	12
Nov	47	13	4	3	4	1	8	14
Dec	35	7	6		2		7	13
Jan	41	2	2	4	1		11	21
Feb	19	11		2			5	1
Totals	211	53	18	13	10	5	46	66

Table 42. Comparative neutralization antibody assays of human sera from patients with Japanese encephalitis.

Patient	Tube Method	Macro	Micro
1. Acute	7.5/300*	<5/300	15/1000
Convalescent	<20/900	<20/300	<20/1000
2. Acute	7.5/300	10/200 <5/300	5/1000
Convalescent	30/300	20/200 <20/200	20/1000
3. Acute	<5/300	<5/200* <5/300	30/70 <5/1000
Convalescent	20/300	40/200** <20/300	80/70* <20/1000
4. Acute	15/300	5/200* 10/800	7.5/70 <5/1000
Convalescent	20/300	40/200* <20/300	80/70* <20/1000
5. Acute	7.5/300	<5/200* <5/300	<5/70* <5/1000
Convalescent	<20/300	60/200* <20/300	40/70* <20/1000
6. Acute	<20/300	<5/200* <20/800	<20/1000
Convalescent	60/300	120/200** 60/300	30/100

* MI titer (recip)/virus dose (LD)

** Retested where possible, increasing serum-virus incubation from 1 to 2 hours.

Table 43. Comparison of homologous serum neutralization titers against chikungunya and Sindbis virus measured by tube, macroplate metabolic inhibition and micro titer metabolic inhibition neutralization tests. Serum virus mixtures incubated at 37 C for one (Sindbis) or two (Chikungunya) hours.

Chikungunya antiserum virus trial.

	Neutralization Method		
Serum	Tube	Macro MI	Micro MI
Rabbit	10/200	160/100*	> 500/70*
Rabbit	10/200	240/100	> 500/70
Mouse	30/200	> 500/100	> 500/70
Human Conv.	> 5/200	10/100	120/70

Sindbis antiserum virus trial

Mouse	7.5/200	> 5/500	15/500
Mouse IAF	10/200	> 5/500	15/500
Mouse	7.5/200	> 5/500	5/500

* Reciprocal of serum titer over virus dose (LD) used.

+ Antiserum to Thailand strain BKM 599.

IAF immune ascitic fluid.

Table 44. Agreement of hemagglutination inhibition (HI) test titers with BHK 21 cell Japanese encephalitis (JE) virus metabolic inhibition test titers of human sera from a dengue epidemic (patients 1 3) and from scattered cases of viral encephalitis.

	HI Test		Micro	Type
Patient No.	Dengue	JE	JE MI	Illness
1	80	20	<5	Undifferentiated
	10,240	20,480	<5	Fever
2	1,280	1,280	20	Dengue like
	5,120	5,120	60	Fever
3	640	2,560	20	Dengue like
	5,120	10,240	30	Fever
4	80	2,560	15	Viral
	160	5,120	30	Encephalitis
5	0	0	0	Viral
	0	0	0	Encephalitis
6	80	1,280	30	Viral
	320	2,500	60	Encephalitis
7	40	640	30	Viral
	20	320	15	Encephalitis
8	0	320	30	Viral
	0	640	30	Encephalitis
9	80	2,560	15	Viral
	640	10,240	60	Encephalitis

* Reciprocal of Titer

Table 45. Proportion of parous *Culex gelidus* collected at Bang Phra, 1966-67.

Month	Number Dissected	Number Parous	Proportion Parous	95% C.L.
March	63	42	.66	.54 - .78
April	198	72	3.6	.30 - .42
May	99	41	.41	.31 - .51
June	157	104	.66	.58 - .74
July	153	69	.45	.37 - .53
August	177	67	.38	.30 - .46
September	168	68	.40	.32 - .48
October	167	79	.47	.39 - .55
November	93	36	.38	.28 - .48
December	148	59	.40	.32 - .48
January	91	37	.40	.30 - .50
February	65	25	.38	.26 - .50

Table 46. Virus isolation attempts from Bang Phra mosquitoes, 1966 1967.

Species	Suckling Mice		Tissue Culture	
	No. Indiv.	Pools*	No. Indiv.	Pools
Culex gelidus	82,169	2/714	51,875	2/367
C. tritaeniorhynchus	75,841	1/602	65,259	0/467
C. fuscocephalus	15,558	0/217	10,566	0/139
Aedes Vexans	5,844	1/75	5,651	1/65
Ae. lineatopennis	4,045	2/51	3,650	2/46
Ae. mediolineatus	3,054	2/51	2,977	1/48
Anopheles aconitus	167	0/5	127	0/4
Mansonia uniformis	660	0/19	454	0/12
M. annulifera	184	0/7	144	0/6

* Pools containing virus/total pools tested

Table 47. Results to date of metabolic inhibition neutralization test screening of reactive wild bird and mammal species from Bang Phra against 30-300 TCLD.. of virus. Only those species reacting are listed.

Sera* from	Virus		
	JE	Sindbis	Chik**
Mammals			
Cyanopterus brachyotis	2/56***	0/56	0/56
Rattus rattus	3/87	2/87	0/87
Rattus norvegicus	1/1	0/1	0/1
Mus cervicolor	2/17	2/17	0/17
Bandicota indica	1/7	0/7	0/7
Birds (all resident species)			
Pycnonotus blanfordi	5/65	0/65	1/65
Pycnonotus goiavier	5/58	0/58	0/58
Passer flaveolus	3/63	1/63	0/63
Rhipidura javanica	3/17	0/17	0/17
Copsychis saularis	1/35	0/35	0/35
Sturnus tristis	1/9	0/9	0/9
Sturnus nigricollis	1/2	0/2	0/2
Rosettus amplexicaudatus	1/1	0/1	0/1
Phragmaticola aedon	0/9	1/9	0/9

* Sera diluted 1:4 - 1:10

** Chikungunya

*** No. of sera neutralizing virus over total sera tested

Table 48. Recovery of arboviruses from *Aedes* mosquitoes from Bang Phra in 1966.

Designation	Date Collected	Species	No. in Pool	Reisol. In
BKM-367/66	14 Jul	*Ae. mediolineatus*	39	Mice, TC*
BKM-448/66	6 Jul	*Ae. mediolineatus*	85	Mice
BKM-457/66	7 Jul	*Ae. vexans*	11	Mice, TC
BKM-589/66	30 Jun	*Ae. lineatopennis*	11	TC
BKM-660/66	20 Jun	*Ae. lineatopennis*	36	TC

*TC LLCMK cell cultures. Primary isolations of all agents were in suckling mice

Table 49. Arbovirus group reaction of two unidentified Bang Phra viruses.

Antigens	Antisera Group A	Antisera Group B
BKM-367/66	<20	640
BKM-589/66	<20	640

Table 50. Hemagglutination-inhibition titers (reciprocals) of mouse anti unknown virus serum against several arbovirus group B antigens.

Antigen (Units)	Antisera BKM-367/66	Antisera BKM-589/66
West Nile (8)	80	80
Japanese Encephalitis (8)	320	640
Tembusu (8)	640	640
Dengue 1 (16)	40	40
Dengue 2 (4)	160	640
Dengue 4 (4)	640	1,280
BKM-367/66	1,280	2,560
BKM-589/66	> 2,560	> 2,560

Table 51. Hemagglutination-inhibition reactions (reciprocals) between two Bang Phra unknown viruses and Tembusu and West Nile viruses.

Antigen (Units)	Antisera BKM-367/66	Antisera BKM-589/66	Antisera Tembusu
BKM-367/66 (8)	640		160
BKM-589/66		1,280	
Tembusu (8)	640	640	> 5,120
West Nile (8)		160	

Table 52. Plaque reduction neutralization tests of three Bang Phra unknown viruses by homologous and other arbovirus group B antisera.

Antiserum (Source)	BKM-367/66	BKM-589/66	BKM-448/66
BKM-367/66 (Mouse)	> 10,240*	> 10.240	
BKM-589/66 (Mouse)		> 10.240	640
Dengue 1 (Monkey)	< 10/300**	< 10/300	
Dengue 2 (Monkey)	< 10/1400		
Dengue 3 (Monkey)	< 10/350		
Dengue 4 (Monkey)	< 10/150		
Tembusu (Mouse)	120/> 1,280	< 10/> 1,280	
Japanese Encephalitis (Rabbit)	< 10/900	< 10/900	< 10/900

* Reciprocal of 50% plaque reducing titer.

** Heterologous titer (reciprocal)/Homologous titer (reciprocal).

Table 53. Results of a serologic survey of horses in Nakorn Pathom.

Horse No.	Age	Serologic Tests				
		Hemagglutination Inhibition			JE	MI
		Chik[1]	Dengue 1	JE[2]	CF[3]	Neut[4]
1	2 yrs	40	0	80	0	5
2	9 mos	0	0	20	0	<5
3	5 yrs	40	0	160	0	5
4	3 yrs	20	40	320	8	5
5	2 yrs	20	0	40	0	<5
6	1 1/2 yrs	0	20	320	0	5
7	1 1/2 yrs	0	0	80	0	10
8	2 yrs	20	0	320	4	10
9	2 yrs	40	0	40	0	<5
10	1 yr	0	0	40	0	<5
11	1 yr	20	20	320	8	7.5
12	1 yr	0	0	60	4	7.5
13	3 yrs	0	0	80	0	7.5

Table 53. (Cont'd)

Horse No.	Age	Serologic Tests				
		Hemagglutination-Inhibition			JE	MI
		Chik[1]	Dengue-1	JE[2]	CF	Neut[4]
14[5]	13 yrs	20	20	2,560	8	80
15	7 mos	0	0	0	0	<5
16[6]	15 yrs	40	20	1,280	8	40
17	6 yrs	40	0	120	0	10
18	9 yrs	320	20	1,280	8	60
19	6 yrs	80	40	1,280	8	60
20	5 yrs	40	40	640	8	60
21	6 yrs	40	40	320	4	10
22	10 yrs	120	0	1,280	8	60
23	9 yrs	40	40	640	8	40
24	9 yrs	80	80	1,280	8	120
25	6 yrs	0	0	640	8	160
26	12 yrs	0	0	320	4	30

Table 53. (Cont'd)

Horse No.	Age	Hemagglutination Inhibition		JE[2]	JE CF[3]	MI Neut[4]
		Chik[1]	Dengue 1			
27	6 yrs	0	40	640	8	20
28[5]	10 yrs	40	40	1,280	16	120
29	3 yrs	20	0	320	0	10

1. Chikungunya Virus
2. Japanese Encephalitis Virus
3. Complement fixation starting at serum dilutions of 1:4
4. Metabolic Inhibition Neutralization vs 300 TC LD_{50} of JE virus
5. Weakness of hand quarters at time of bleeding.
6. Aborted in September in 5th month of gestation.

Table 54. Serological response (reciprocal titers) to 3 arbovirus antigens in a horse with clinical encephalitis

Sampling Date	Hemagglutination Inhibition			JEV Complement Fixation	JEV Neut. Metabolic Inhibition
	Chik	Dengue 1	JEV		
10 Nov 66	20	<20	40	0	<5
17 Nov 66	40	<20	640	4 8	60

SEATO Clinical Research Study on Bladder Stone*

Coordinator: Aree Valyasevi, M.D. — Chief, Thai Component Clinical Research Center

Principal Investigators:

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Thara Viriyapanich, B.Sc. (in Pharm)

Period of Report: 1 April 1966 - 31 March 1967

GENERAL INFORMATION

The results of a series of investigations from this Laboratory comprising 8 papers, were recently submitted for publication in the American Journal of Clinical Nutrition[1-8]. The studies suggested that vesical calculus formation is a neo-natal disease and that the nutritional status of the mother fetus somehow contribute to the development of uroliths. The most pertinent findings can be summarized as follows:

1. Infant feeding practices differ markedly between families living in villages (hyper-endemic area) and in Ubol City (hypo-endemic area). Essentially all newborn in both locations are breast-fed. About 60% of village mothers started their infants on supplemental glutinous rice feedings during the first week of life, usually after the 3rd day. In the city, on the other hand, only 8% of the mothers stated they fed infants supplemental foods during the first 4 weeks of life and only 52% of the infants received rice during their first 3 months. If caloric requirements for infants are calculated on the basis of 115 ± 15 calories/Kg body weight/day, the glutinous rice fed village infants could supply about one-half their total daily requirement.

2. It was a fairly general observation that village women during pregnancy and lactation did not increase their intake of proteins or of foods in general. Deviations from their usual diets were only in the direction of greater food restrictions, particularly during the third trimester. This was done in the belief that it would result in a smaller infant and an easier delivery.

3. Twenty-four hour urine volumes were frequently less in village than in city newborn infants under 1 year of age, and in children 2 to 10 years old. This was not always observed but varied with season and location. The urinary osmolarity was generally lower in the village samples than in those from the city, and the total number of osmoles excreted in 24 hours was significantly lower in the village newborn less than 15 days old.

* Supported in part by USPH Grant NIH A-5921 given to Dr. Paul Gyorgy

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4. Significantly lower urinary phosphate concentrations and 24 hour excretion values were found in village samples of all age groups up to 1 year old compared with the city Values. On the contrary, the urinary calcium concentration and 24 hour excretion value were somewhat higher in the village than in the city samples in the newborn age group. No differences could be demonstrated in the older age groups.

5. The 24 hour excretion of magnesium and of uric acid were very similar in both locations in all age groups up to 10 years of age. Oxalate excretion was also very similar in the subjects studied.

6. The total inorganic sulfate and the free inorganic sulfate in the urine were lower in village samples from boys up to 1 year of age than in city samples. This was true whether the data were expressed on the basis of concentration, 24-hour excretion, or related to creatinine excretion.

7. Oxalate crystalluria was observed in 12 of 28 village boys under 45 days of age. On the contrary, none of the 39 city infants of the same age group had oxalate crystalluria. Uric acid crystalluria was found equally in both village and city samples.

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1. Halstead, S.B., and Valyasevi. A.: Studies of Bladder Stone Disease in Thailand. I. Introduction and description of studied area. American Journal of Clinical Nutrition.

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8. Van Reen, R., Valyasevi, A., and Dhanamitta, S.: Studies of Bladder Stone Disease in Thailand. VIII. Sulfate excretion by newborn and infants in three localities: the possible relationship of protein nutrition to vesical lithiasis. American Journal of Clinical Nutrition.

STUDY REPORTS

1. Title: "Effect of orthophosphate, fat-free powdered milk, methionine and Vitamin B_6 supplements on the occurence of crystalluria and excretion of urinary constituents"

Principal Investigators; Sakorn Dhanamitta, M.D.
Aree Valyasevi, M.D.
Robert Van Reen, Ph.D.

Assistant Investigator Thara Viriyapanich, B.Sc. (in Pharm)

Objectives

To determine the influence of a variety of dietary supplements upon the urinary excretions of phosphate, sulfate sulfur, calcium, oxalate, uric acid, magnesium, sodium, potassium, and chloride.

Description

Twenty five male infants ranging in age from 6 to 19 months residing in 3 villages in Ubol Province were included in the study. A fairly high incidence of bladder stone disease among the inhabitants of these villages had previously been established.

Two forms of phosphate supplementation, inorganic (mixture of KH_2PO_4 and Na_2HPO_4) and organic (fat free powdered milk), were administered. Each supplement provided approximately 600 mg of phosphorus dialy. A latin square design was employed so that each subject received supplements of orghophosphate, milk and control regimen. The sequence of supplementation was altered in different subjects to include the 6 possible combinations. Each supplement was given for 6 days before changing to a different supplement.

Twenty four hour urine collections were made during the last period of giving each supplement, and freshly voided early morning urine samples were collected each day by utilizing pediatric urine-collecting bags as previously described. Qualitative tests for pH, protein and sugar were made by $COMBISTIX_R$ paper strip. Microscopic examinations were performed daily on centrifuged, freshly voided urine samples within two hours of collection to determine the accurrence of crystalluria and other possible abnormalities.

Nineteen of the same group of village infants used for the phosphate study were given supplements of DL-methionine (300 mg per day), vitamin B_6 (3 mg per day) and placebo in an experiment of similar design, but carried out 6 weeks earlier. In this study, the supplements were mixed in small quantities of 80% sucrose and fed once daily. The sucrose solution alone was used during the control period.

All subjects were fed on breast milk and premasticated glutinous rice except one subject, whose mother died, was fed on cow's milk formula,

Progress

Qualitative tests for urinary protein and sugar were normal. The number of leucocytes or erythrocytes in the centrifuged urine samples was found to be less than five per high power field.

The occurrences of oxalate and uric acid crystalluria during the course of study are shown in Tables I and II. Four of 21 subjects of the phosphate trial did not show any oxalcrystalluria at any time during the study. One subject who was on an artificial milk formula, showed no cystalluria during the course of 30 microscopic examinations. The data in Tables I and II are presented in terms of the number of occurrences of crystalluria and the number of examinations made. It can be seen that when the infants received placebo, methionine or vitamin B , 23 to 29 percent of urine examinations revealed oxalcrystalluria, whereas none of the samples from infants receiving orthophosphate demonstrated oxalate crystals. Disappearance of the oxalcrystalluria usually occurred within 24 hours after the supplementation of phosphate.

The data suggest that milk supplementation is not as effective as orthoyhosphate in reducing the occurrence of oxalcrystalluria. However, it should be pointed out that all subjects rejected part of the milk in the first few days, some developed loose stools and the period of study war only six days. Therefore, it is possible that the amount of phosphate absorbed might be less from milk than from the orthophosphate supplementation.

A total of 60 urine examinations were also carried out on 4 infants whor were supplemented with milk feedings for 18 consecutive days. A total of 5 occurrences of oxalcrystalluria were observed. Three occurrences were found in one infant who had mild recurrent diarrhea and two occurrences were during the first two days of supplementation in another infant.

The data presented in Table I on oxalcrystalluria are totals of observed occurrences of crystalluria without regard to the sequence of supplementation. It is possible that when inorganic orthophosphate was administered there might be a carry-over into the next period of supplementation. To check this, the data were recalculated to determine the number of occurrences of oxalcrystalluria in infants receiving the placebo either before or after the orthophosphate supplementation. In the control group prior to phosphate administration, there were 14 occurrences out of 33 examinations or 42 percent. In the control group after the phosphate supplementation, there were 6 occurrences of oxalcrystalluria out of 35 examinations or 17 percent. It thus appears that there is a carry-over of the phosphate effect into the next supplementation period. No such carry-over effect was obvious in the cases of infants receiving milk after phosphate or placebo after milk. However, the number of cases in the present series is too small to draw any firm conclusions concerning carry-over effects and an experiment will be designed to test this.

The mean urine pH rose from 5.9 during the control period to 7.1 during the orthophosphate supplementation. To test whether there is a relationship between the alkalinity of urine and disappearance of the oxalcrystalluria, at the end of the regular study period, five infants were administered sodium bicarbonate (2 to 3 gm/day) for 6 days. A rapid rise in the urinary pH values was obtained but 9 out of 30 urine examinations still demonstrated oxalate crystals. The findings do not support the concept that alkalinity of the urine alone is involved in the disappearance of oxalcrystalluria.

Uric acid crystalluria (Table II) also appears to be reduced by supplementation with either orthophosphate or fat-free powdered milk, although uric acid crystals were not completely eliminated by phosphate, as in the case of oxalcrystalluria. It is possible that alkalinity of the urine was a factor in reducing the uric acid crystalluria during phosphate supplementation. However, no change in urinary pH was observed during the milk supplementation, therefore, further study will be required before any conclusion can be drawn. There was no obvious effect of methionine or vitamin B supplementation on uric acid crystalluria.

From the data presented above it appears that the oral administration of inorganic orthophoshate will eliminate the oxalcrystalluria and probably reduce the uric acid crystalluria commonly found in village urine samples. Oral administration of DL-methionine and vitamin B have no such effects. It must be determined in further studies whether the effect is a specific response to phosphate or whether other supplements will act in a similar manner.

The question immediately arises as to the mechanism(s) by which phoswhate exerts its effect. It is known that dietary inorganic phosphate will reduce the absorption of calcium from the gastro-intestinal tract. In the present study, phospate supplementation had a rapid effect on oxalcrystailuria, thus suggesting some other mechanism of action. Vermeulen et al[1] and Miller et al[2] have indicated that many normal urinary components, such as citrate, urea, K, Na, SO_4, PO_4, Cl and Mg ions are all effective in increasing the solubility of calcium phosphate and calcium oxalate in water. It is expected that the phosphate and milk supplementations of the present study resulted in increased urinary excretion of phosphate.

Fleisch and Bisaz[3] have indicated that pyrophosphate will inhibit both hydroxyapatite and calcium oxalate precipitation. Furthermore, Fleisch and Bisaz[4] have demonstrated that the oral administration of orthophosphate to healthy subjects induces a significant increase in urinary pyrophosphate excretion. The determination of the concentrations of the various urinary components and ions mentioned above is now underway.

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Table 1

Occurrence of oxalcrystalluria in village infants following supplementation with a variety of substances

Supplement	No. of Infants	Oxalcrystalluria		
		No. of occurrences	No. of examinations	No. of infants with crystalluria
Placebo	17*	25	93	17
Orthophosphate	17	0	83	0
Milk	17	7	87	5
Placebo	14**	20	70	7
Methionine	14	20	70	10
Vitamin B_6	14	16	70	11

* There were 17 of the original 21 infants who showed oxalcrystalluria at some time during the study.

** There were 14 of the original 19 infants who showed oxalcrystalluria at some time during the study.

Table II

Occurrence of uric acid crystalluria in village infants following supplementation with a variety of substances

Supplement	No. of Infants	Uric acid crystalluria		
		No. of occurrences	No. of examinations	No. of infants with crystalluria
Placebo	15*	22	83	15
Orthophosphate	15	6	73	3
Milk	15	10	77	5
Placebo	16**	11	80	7
Methionine	16	18	80	11
Vitamin B.	16	12	80	8

* There were 15 of the original 21 infants who showed uric acid crystalluria at sometime during the study.

** There were 16 of the original 19 infants who showed uric acid crystalluria at some time during the study.

2. Title: "Study of Urinary Mucosubstances"

Principal Investigators:	Aree Valyasevi, M.D. Sakorn Dhanamitta, M.D.
Assistant Investigators:	Jaratbhan Yooktatat, B.Sc. (in Pharm) Potjanee Threeratana, B.Sc. (in Pharm)

Objectives

To study the urinary total non-dialyzable solids and their subfractionation in infants of hypo-and hyper-endemic areas.

Descriptions

Twenty-four hour urine samples were collected from male newborn and infants ranging in age from 3 days to 12 months.

Urine collections were made from infants living in Ubol villages, Ubol, and Bangkok. A Urine collection bag was used and after each voiding, the bag was emptied into the container which was always kept at a temperature of 1° to 5°C. The CRC nurses supervised the collection at all times. Repeated collections were performed on the following days if the first collection was incomplete.

The amount of the total non-dialyzable solid (TNDS) was determined by taking dry weight at 90-100°C for 1-1½ hours of the non-dialyzable urine after three day cold dialysis against demineralized water. The rest of the non-dialyzable urine was lyophilized and measured for TNDS by method according to Boyce and King (J. Clin. Invest. 37:315, 1958.) Determination of the TNDS by lyophilization has been completed on only one-half of the total number of urine samples. Thus far, the results (by lyophilization) are comparable to the dry weight method.

Subfractionation of the urinary mucosubstances was determined by Gel Filtration as described by Gale, Cornelius and Bishop. [1] The total salt-soluble proteins and Tamm-Horsfall (T-H) mucoproteins were measured at 280 m/u using a Backman DU spectro-photometer. The results were expressed as ODU per 24 hours.

Progress

The mumber of subjects, mean ages, urine volumes and average pHs in different locations and age groups are shown in Table I. The village 24-hour urine volume was significantly less ($0.05 < P < 0.10$) than the Ubol cit, and Bangkok volumes in the 3 to 15 day old group.

The village newborn excreted significantly higher amount of TNDS than those excreted by city newborn (Table II). Infants (older than 30 days) from both city and villages show no significant difference of TNDS excretion. When these values are compared with the Bangkok and the U.S. groups. There TNDS excretion was less in the village subjects when compared to values obtained from the subjects from cities of Ubol and Bangkok and in infants over 30 days old.

Table IV shows the daily urinary excretion of Optical Density Units (ODU) of salt-soluble proteins and Tamm-Horsfall (T-H) mucoprotein in newborn and infants of Ubol villages and city. The salt-soluble protein excretions were significantly higher in samples from villages than those from city in the three age groups (3-15 days, 16-30 days, and 7-12 months).

The newborn and infants of all age groups from villages also excreted a consistently higher amount of T-H mucoprotein than those from the city. Marked decreases in the ratio of Salt Soluble Protein and T-H mucoprotein were also observed in village infants, over 15 day old. These findings demonstrated a strikingly higher excretion of the T-H mucoprotein. Boyce and Swanson[2] also observed high salt-insoluble protein (T-H mucoprotein) excretion in the urine of patients with renal calculous disease. Therefore, our findings may be significant.

Table V demonstrates the percentages of various molecular weight groups of salt-soluble urinary proteins in village and Ubol city infants. Significantly higher excretion in the 5,000 to 10,000 and the 10,000 to 100,000 molecular weight fraction of salt-soluble urinary proteins was observed in the 1,000 to 5,000 molecular weight fraction was observed in the city infants. No difference is observed in the 100,000 molecular weight fraction.

Our results are comparable with those of Mia and Cornelius[3] who demonstrated a significant daily increase in the 4,000 to 9,000 molecular weight fraction of salt-soluble urinary proteins in sheep receiving a "calculi-provoking" diet as compared to normal sheep.

In summary, the village infants excreted higher urinary TNDS, T-H mucoprotein and 5,000 to 10,000 MW fraction of salt-soluble proteins than the city subjects. The significance of these findings to stone formation requires further study.

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Table 1

Numbers and mean ages of subjects, and 24-hour urine volumes and pHs, 1965–1966

Age and Locality	No. of subj.	Mean age	Urine vol. ml.	Average pH
3–15 days				
Village	10	9	85 ± 22	6.0
Ubol City	12	9	142 ± 20	6.1
Bangkok	20	5	146 ± 18	6.6
16–30 days				
Village	14	22	236 ± 26	6.4
Ubol City	17	24	239 ± 27	6.0
Bangkok	3	21	304 ± 40	6.8
1–6 months				
Village	46	2.7	244 ± 17	6.4
Ubol City	38	2.7	261 ± 20	6.0
Bangkok	21	3.6	290 ± 25	6.9
7–12 months				
village	16	9.5	205 ± 16	6.6
Ubol City	17	9.5	242 ± 41	6.8
Bangkok	19	10.2	174 ± 19	6.4

Table II
Urinary Total Non dialyzable Solids (TNDS)* in Newborn and infants

Ages	Villages		City	
	No. of subj.	TNDS mg/24 hrs	No. of subj.	TNDS mg/24 hrs
1.15 days	10	130.0 ± 21.7**	12	57.8 ± 9.6
16.30 days	14	120.5 ± 20.1	17	86.6 ± 7.9
1.6 months	46	114.0 ± 8.5	38	110.2 ± 9.6
7.12 months	16	146.6 ± 14.1	17	152.3 ± 27.7

* TNDS as Determine by Total Dry Weight.
** P < 0.01 (Villages compare with City)

Table III

Urinary Total Non Dialyzable Solids (TNDS) by
Dry Weight in Different Geographic Area

Area	Newborn		Infants	
	No. of subj.	TNDS (mg/24 hr)	No. of subj.	TNDS (mg/24 hr)
Ubol Villages (V)	24	124.46 ± 14.50	62	122.39 ± 7.47
Ubol City (C)	29	74.69 ± 6.56	55	176.00 ± 10.98
Bangkok (B)	23	61.64 ± 9.83	40	155.86 ± 14.13
U.S.A.*	7	89.9 ± 26.8	3	124.5
p V vs C		< 0.01		< 0.01
V vs B		< 0.01		0.05 - 0.02
C vs B		0.50 - 0.10		0.50 - 0.10

* Keutel and King: Clin. Chem. Acta II (1965)

FIGURE I

Percentage of various molecular weight groups of salt-soluble urinary proteins in male infants

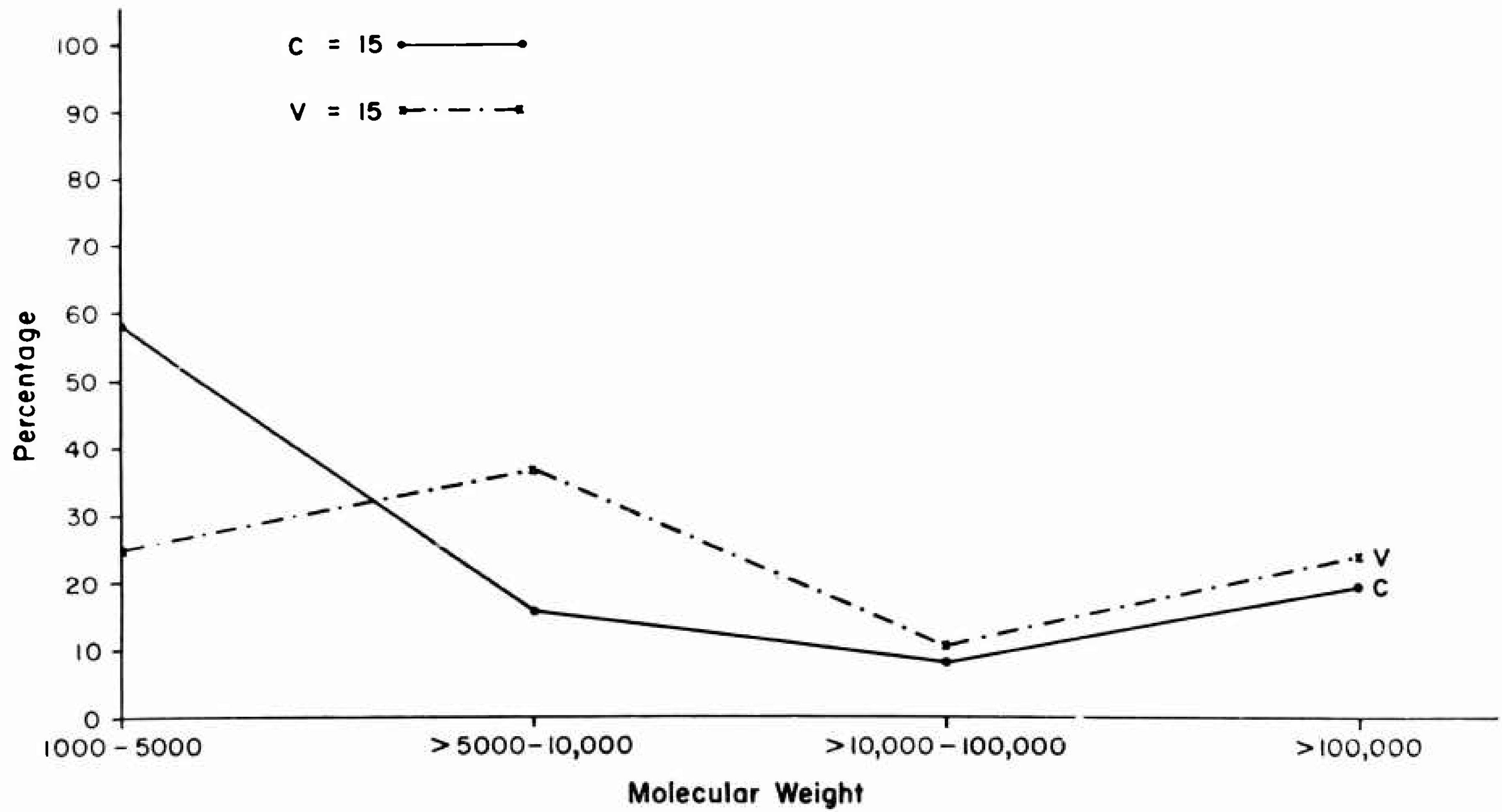

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Table IV
Daily excretion of Optical Density Units (ODU) of salt soluble urinary protein* and Tamm-Horsfall (T-H) mucoprotein** in male newborn and infants

Age	Salt Soluble Protein ODU/24 hrs		T-H Protein ODU/24 hrs		Ratio: SSP / T-H	
	Village	City	Village	City	Village	City
3-15 days	(2) 78.6 ± 7.3[d]	(8) 47.6 ± 12.3	(2) 2.9 ± 1.9	(8) 1.7 ± 0.4	1:0.03	1:0.03
16-30 days	(8) 87.6 ± 10.1[a]	(13) 52.9 ± 7.2	(8) 75.2 ± 73.5	(13) 1.6 ± 0.2	1:0.86	1:0.03
1-6 months	(30) 51.3 ± 3.9	(26) 65.1 ± 14.6	(30) 48.2 ± 30.3	(26) 20.0 ± 14.4	1:0.94	1:0.31
7-12 months	(5) 101.4 ± 17.5[d]	(6) 39.9 ± 4.5	(5) 103.3 ± 94.6	(6) 15.2 ± 7.7	1:1.02	1:0.38

* Salt soluble proteins equal RS_1
** (T-H) Mucoprotein equal R^1

Probability value	Village with City
$p < 0.01$	a
$0.05 < p < 0.10$	d

Table V
Percentage of various molecular weight groups of salt-soluble urinary proteins in male infants from Ubol Villages and Ubol City

Area	Moleculer Weight (MW) Groups-Percent of Total in 24 hrs Sample					
	Urine Samples Number	Mean Ages months	1,000-5,000 MW %	5,000-10,000 MW %	10,000-100,000 MW %	100,000 MW %
Villages	15	5.0	24.95 ± 6.11[a]	36.71 ± 5.06[a]	15.01 ± 3.28[d]	23.28 ± 2.03
City	15	5.02	58.03 ± 6.44	15.04 ± 3.49	7.97 ± 1.22	19.11 ± 3.36

Probability Value	Village with City
$p < 0.01$	a
$0.05 < p < 0.10$	d

3. Title: Study of Organic Matrix of Stone.

Principal Investigators: Sakorn Dhanamitta, M.D.
Aree Valyasevi, M.D.

Assistant Investigator: Jaratbhan Yooktatat, B.Sc. (In Pharm)

Objectives

To study the organic matrices of stones from patients in various parts of Thailand.

Description

Thus far, 21 bladder stones obtained from patients, aged 1 to 5 years, have been studied.

Isolation of the matrix components, demineralization and fractionation were accomplished by the method described by Keutel[1]. The stones were powdered and demineralized with 5% EDTA solution for 3 days, then with 0.1 M Veronal (pH 8.6) for 24 hours and with weak basic solution (pH 11.4) for 2 hours. under continuous stirring. After demineralization, they were dialyzed against alkaline solutions and demineralized water. Ten percent of the solutions was lyophilized and determined for the total organic matrix. The rest was analyzed for the subfractions of the matrix, using the method described by Keutel.

Identification of the Amino Acids and Carbohydrate Component was also performed by chronato-graphic technic as described by King and Boyce[2].

Progress

Table I shows that organic matrix comprised from 1.7 to 3.3 percents of the total weight of stones. Ammonium urate and calcium oxalate were the main chemical compositions of stones. The organic matrix contents did not seem to correlate with either the age of patients or the mineral compositions. None of our stones was free from the organic matrix.

The percentage of matrix by weight was reported by Gasser et al[3] as 3 to 5%, by Philipshorn[4] as 2 to 3.5%, by Boyce and Garvey[5] as averaging 2.87%, and by Keutel[1] as averaging 2.75% for the inorganic-crystalline stones and 1.06% for the organic-crystalline stones. The present results are comparable to those of renal stone reported previously.

The preliminary results of Amino Acids detectable by chromatography in one stone, obtained from a male patient aged 5 years, are presented in Table II. Chromatographically, 5 carbohgdrates were also identified. These included galactose, glucose, mannose, rhamnose and one unidentified spot. These results are similar to the composition of calculi reported by King and Boyce[2]. Further study is required before any conclusion can be drawn.

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Table I
Isolation of Organic Matrix of Stones

No. of Subjects and Stones	Average age years	% matrix	Crystal Composition					
			Am. Urate	Ca Ox	$CaPO_4$	$MgNH_4\ PO_4$	Ca Carb	Mg Carb
3	1 yr	3.31	4^+	1^+	1^+	1^+	trace	trace
5	1 – 2	1.65	4^+	1^+	1^+	1^+	trace	1^+
5	2 – 3	2.21	4^+	3^+	1^+	1^+	1^+	2^+
6	3 – 5	2.93	4^+	3^+	2^+	2^+	1^+	1^+
2	unknown	2.15	4^+	3^+	—	—	—	—

Table II
Amino Acids Identified Chromatographically

Alanine	Glycine
Glutamic acid	Threonine
Aspartic acid	Lysine
Serine	Proline
Leucine	Tryptophane
Isoleucine	Tyrosine
Valine	Methionine
Phenylalanine	Arginine

4. Title: Study of urinary hydroxyproline excretion in infants of hyper and hypo endemic areas.

Principal Investigators: Aree Valyasevi, M.D.
Sakorn Dhanamitta, M.D.

Assistant Investigator: Pichai Thuvasethakul, B.Sc. (in Pharm)

Background

There is fairly good evidence to support the suggestion that urinary hydroxy proline arises from the breakdown of collagen[1]. A reduction in the amount of insoluble collagen in the malnourished infant could result in a decrease in urinary hydroxyproline excretion[2,3].

Urolithiasis has been produced in weanling rats fed a diet low in protein and is prevented by increasing the protein intake with ceither casein or soybean protein[4]. A previous study showed that total inorganic sulfate and the free inorganic sulfate in the urine were lower in samples obtained from village boys up to 1 year of age than in city subjects of a similar age range. These findings may indicate a lower sulfur containing amino acids intake among the village infants. It therefore, seemed worthwhile to the protein nutritional status of infants and young children residents of Ubol city with a comparable group of village subjects.

Objectives

To compare the urinary hydroxyproline and creatinine excretions in newborn and infant of hyper endemic area (villages) with those of hypo endemic area (city and Bangkok).

Description

Urine samples collected for urinary mucosubstances determination were also used for this study. The detailed information regarding subjects, method of collection, 24 hour volume and pH, have been previously described. The hydroxyproline determination was performed using the method described by Prockop and Udenfriend[5]. The urinary creatinine was determined using the standard automated technic.

Progress

Table I and II show the mean urinary creatinine and hydroxyproline excretions in different age groups and locations. The hydroxyproline excretion in Ubol newborn and infants (1 6 months) is higher than in subjects from Bangkok when expressed per mg. creatinine. This is probably due to the low creatinine excretion in Ubol subjects as compared to Bangkok subjects. The Village and city subjects appeared to excrete more total hydroxyproline than the Bangkok group; however, no statistically significant difference could be demonstrated. It is also of considerable interest to note that total hydroxyproline excretion is similar in all age groups except in the newborn, 1 to 15 day old.

Urine hydroxyproline is increased in growing children and in patients with acromegaly and decreases when growth stops, as noted in adults and treated acromegalic patients. It was also demonstrated that the amount of urine hydroxyproline is influenced considerably by diet; the excretion increased when the

subjects were fed gelatin rich foods. Since our subjects received a regular diet which is somewhat different from that ingested by Ubol and Bangkok subjects, the present data may not necessarily indicate a similar nutritional status among these infants.

Urine creatinine excretion is mainly related to a muscle mass, especially in growing children and patients recovering from malnutrition[illegible]. Our creatinine excretion data from Bangkok infants (under 7 month old) are slightly higher than from those in Ubol. This observation may reflect a better nutritional status of Bangkok infants than those in Ubol. However, further studies are required before any definite conclusions can be reached.

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Table I
Mean Urinary Creatinine Excretion in Different Age Groups and Location

Location	No.	1-15 days	No.	16-30 days	No.	1-6 months	No.	7-12 months
Mg%								
Villages	10	21.9 ± 4.4	14	9.8 ± 0.8	46	15.6 ± 1.1	14	28.5 ± 3.4
City	12	10.4 ± 0.9	17	10.3 ± 0.7	38	15.6 ± 2.8	17	26.9 ± 4.4
Bangkok	20	18.1 ± 2.8	3	9.3 ± 1.7	21	17.0 ± 3.1	19	22.8 ± 3.0
Mg/24 hrs								
Villages	10	14.5 ± 2.4	14	21.7 ± 1.8	46	33.9 ± 1.8	14	56.0 ± 5.9
City	12	14.6 ± 2.2	17	21.3 ± 1.4	38	32.5 ± 2.3	17	44.9 ± 4.2
Bangkok	20	22.5 ± 2.8	3	29.5 ± 8.4	21	42.5 ± 5.3	19	41.3 ± 6.1

Table II
Mean Hydroxproline Excretion in Different Age Group and Location

Location	No.	1-15 days	No,	16-30 days	No.	1-6 months	No.	7-12 months
Mg+								
Villages	10	10.72 ± 2.10[cy]	14	8.45 ± 1.11[y]	45	8.07 ± 0.96	16	7.88 ± 0.87
City	10	5.83 ± 0.66	18	8.06 ± 0.83[o]	38	7.71 ± 0.86[o]	15	7.58 ± 1.27
Bangkok	16	5.47 ± 0.96	3	5.12 ± 0.96	21	5.30 ± 0.53	16	6.48 ± 1.07
Mg/24 hours								
Villages	10	7.19 ± 1.48	14	19.02 ± 2.65	45	17.25 ± 1.48	16	15.04 ± 0.96
City	10	8.03 ± 1.29	18	16.23 ± 1.42	38	17.19 ± 1.24	15	16.98 ± 2.73
Bangkok	16	6.95 ± 1.18	3	15.33 ± 3.15	21	16.11 ± 1.96	16	11.35 ± 2.01
Mg/mg creatinine								
Villages	10	0.504 ± 0.073[y]	14	0.910 ± 0.111	45	0.545 ± 0.053[z]	16	0.269 ± 0.027
City	10	0.601 ± 0.077[m]	18	0.771 ± 0.056	38	0.581 ± 0.050[o]	15	0.401 ± 0.113
Bangkok	16	0.293 ± 0.045	3	0.630 ± 0.240	21	0.411 ± 0.054	16	0.250 ± 0.022

Probability Value	Village with City	Village with Bangkok	City with Bangkok
$P < 0.01$	a	w	m
$0.01 < P < 0.02$	b	x	n
$0.02 < P < 0.05$	c	y	o
$0.05 < P < 0.10$	d	z	p

SEATO MEDICAL RESEARCH STUDY ON CHOLERA

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Period of Report: Annual, 1 April 1966–31 March 1967

Objective–The objective of this study is to further our understanding of the pathogenesis and immunology of cholera.

Description–Efforts during the year have been directed primarily to an expansion of our knowledge concerning the production, mode of action, and immunology of "choleragen," an antigenic, choleragenic, protein moiety elaborated *in vitro* by certain strains of cholera vibrios. In addition, an effort was made to develop a more effective conventional cholera vaccine based upon a new method of examining the antigenicity of candicate strains.

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Progress:

1. Production of choleragen in a simple, completely defined medium.

The present method for the production of choleragen involves the growth of *V. cholerae* 569 B in a synthetic medium supplemented with casamino acids, an un defined mixture of amino acids derived from the hydrolysis of casein. This apparent requirement of casamino acids has been confirmed in several laboratories. If choleragen could be produced in a completely defined medium, it might facilitate the ultimate purification of this material and it might, at the same time, enable the production of choleragen in greater yield. We had previously attempted to substitute a mixture of amino acids, based on an analysis of the casamino acid medium, but failed to obtain detectable choleragen production. In view of the present importance of this problem, it was decided to attempt again the production of choleragen in a completely defined medium. Accordingly, a battery of media was prepared consisting of the simple basal medium with various supplements; a mixture of the L amino acids found in casamino acids (we had previously used DL amino acids), ash from casein, a vitamin mixture, a complete tissue culture medium, and various combinations of the supplements. *V. cholerae* 569 B was cultivated in the various media in the usual manner (with an inoculum of 10^3 10^4 cells per ml at 37 C in fairly shallow layers with shaking) and filtrates were prepared and tested for choleragenicity in infant rabbits. It was found that substitution of a mixture of 16 L amino acids for casamino acids allowed production of choleragen in the basal medium. The following amino acids and amounts were used:

Amino acid	mgm%
Aspartic acid	19
Threonine	12.5
Serine	16
Glutamic acid	59
Proline	20
Glycine	7
Alanine	10
Valine	17.5
Methionine	8
Isoleucine	12
Leucine	30
Tyrosine	5
Phenylalanine	11.5
Lysine	30
Histidine	10
Arginine	12

Subsequent tests, employing pools of individual amino acids, have yielded variable and inconsistent results. Additional study to determine the optimal conditions for satisfactory yields of choleragen is in progress.

The Department of Biologics Research, WRAIR, has undertaken the production of larger batches of crude choleragen which have been forwarded to this laboratory for further purification. Thus far, four lots have been received and three of these have been processed. The first was relatively inactive, but purified choleragen has been obtained from the other two processed. The yields have been lower than might be expected from previous experience with pools of small batches of crude filtrate.

The majority of cholera strains which have been tested failed to elaborate choleragen *in vitro* (under the conditions we employ for choleragen production from strain 569 B) although most of the same strains are of proven choleragenicity in the intra-intestinally infected infant rabbit. To test the possibility

that failure to elaborate detectable choleragen *in vitro* may be due to its inactivation or breakdown by non-elaborating strains, a known amount of choleragen (20 ug/ml) was added to a Syncase broth culture of a non-elaborating strain (NIH 35, Inaba) and, in parallel, to sterile medium and to a culture of 569 B. After 18 hours at 37°C, filtrates were prepared and tested in infant rabbits. The culture filtrate from NIH 35 without added choleragen was, as expected, not choleragenic; the filtrate with added choleragen was choleragenic as were the filtrates of sterile medium with added choleragen and the filtrate of 569 B with choleragen. Thus, tests with this single strain suggest that *in vitro* inactivation of choleragen is not the reason for the failure of "non-elaborating" strains to produce choleragen *in vitro.*

2. Further purification of choleragen on ion-exchange resins.

Previous study of "purified choleragen" (choleragen which was passed through Sephadex) by disc electrophoresis indicated that multiple protein components were presents: as many as 11 protein-staining bands could be detected by this sensitive technique, with only one area associated with choleragen antigen as demonstrated by immune precipitation. Accordingly, an attempt was made to further purify choleragen by means of ion exchange. A 5 mg sample of choleragen was applied to a DEAE cellulose column in 0.001 M phosphate buffer at pH 6.4 and fractions were collected following increases in the buffer concentration to 0.01 M and 0.3 M. The fractions were dialyzed and lyophilized and tested for choleragen content by the Ouchterlony technique, in infant rabbits, and by skin testing in adult rabbits. Essentially all the activity was found in the fraction eluted by 0.3 M buffer. The results indicate that it is feasible to achieve a further degree of purification of choleragen by ion exchange chromatography.

Sephadex purified choleragen has been analyzed by the sucrose density gradient centrifugation technique. Fractions collected after sucrose gradient centrifugation (10–40 % at 100,000 g for 18 hours) were tested for choleragen by the Ouchterlony agar gel double diffusion immune precipitation technique with specific anticholeragen serum. The choleragen antigen was found primarily in fractions 6 and 7 indicating that it has a sedimentation coefficient approximating 6–7 S.

Additionally, an effort is being made to obtain disc electrophoretically pure choleragen by the technique of preparative disc electrophoresis. Preliminary attempts to separate serum proteins by this method have suggested the feasibility of its application to the ultimate purification of choleragen. When, and if, pure choleragen becomes available it should be possible to determine its precise chemical composition and gain further insight into its mode of action.

3. Choleragen elaboration in vivo.

In order to learn more regarding the elaboration of choleragen *in vivo* and its relation to the pathogenesis of experimental cholera, a series of experiments was designed to determine if, when, and in what amount, choleragen was produced in *V. cholerae* 569 B infected ligated ileal loops of adult rabbits, and to compare the response to infection with that following inoculation of purified choleragen.

Initially, a "standard curve" of the response of isolated loops to varying doses of choleragen, in terms of milliliters of fluid elaborated per centimeter of intestine, was constructed for comparison and for use as an additional bioassay of choleragen. This curve is similar to that described by Burrows and Musteikis (J. Inf. Dis. 116: 183, 1966) for cholera "whole cell lysate" with the exception that purified choleragen is approximately 200 times more active in this model. The time sequence of outpouring of fluid in response to choleragen was also studied. From the results, it appears that fluid accumulation begins to appear at about 2 hours, the earliest time when an increase in vascular permeability was detectable in the infant rabbit model, and then proceeds at a rate and to a maximum volume which is determined by the amount of choleragen injected initially.

Ligated loops were infected with varying doses of living vibrios and the sequence of changes was followed; viable counts were determined and the choleragen (equivalent) content was determined by the rabbit skin assay. Fluid began to accumulate at about 6 hours, at a time when the vibrio population was increasing logarithmically from inocula of 10^6 or 10^8 vibrios. An inoculum of 10^4 vibrios only occasionally gives rise to a "positive loop", in our hands. Choleragen is also elaborated during the development of the "positive loop" and continues at a rapid rate until 18 hours when it tapers off coincident with the maximum stationary phase of the bacterial growth. The choleragen content of loops inoculated with 10^6 and 10^8 vibrios was, at 18 and 24 hours, sufficient according to this assay to yield a precipitin band when 0.05 ml of 20X concentrated intestinal fluid was placed in Ouchterlony wells opposed to anti-choleragen serum. When 20X concentrates of each of the intestinal fluids were tested in this manner, only the 18 and 24 hour fluids from those inocula were positive. Additional assays of the choleragen content of the loop fluids using the rabbit loop assay are in progress.

To determine whether other strains elaborate choleragen *in vivo*, fifteen strains of cholera vibrios were inoculated, in doses of 10^8 organisms, into ligated loops of small intestine of adult rabbits. Of these, all but two were previously demonstrated not to elaborate choleragen *in vitro*. After 18 hours, the fluids were collected, centrifuged, filtered, lyophilized and resuspended in 1/20th of the original volume. The intestinal fluid concentrates were then tested for the presence of choleragen by the Ouchterlony technique using specific anti-choleragen antiserum. In addition, the filtrates were diluted serially and tested by intradermal inoculation of adult rabbits for their ability to produce typical skin reactions. Choleragen was demonstrated immunologically in intestinal loop fluid filtrates of 4 strains. Of these, two, *V. cholerae* 569 B Inaba and El Tor Ogawa 3083-13 had previously been shown to elaborate choleragen *in vitro*. The two additional strains, *V. cholerae* Ogawa VC12RX1 and *V. cholerae* Inaba 16, elaborated choleragen *in vivo* but not *in vitro* under the conditions previously employed. Those filtrates which contained immunologically detectable choleragen tended to exhibit higher titer skin reactivity than did the other filtrates. The reason why choleragen could not be detected with the other choleragenic vibrios remains to be determined. However, since, as will be shown below, amounts of choleragen less than those which can be detected immunologically provoke the positive loop reaction, it is entirely plausible that sufficient choleragen is released early in the infection to bring about the outpouring of fluid which then dilutes the choleragen to below detectable levels.

4. Histological observations on isolated loops

Histologic observations were made on intestinal tissue taken from adult rabbits sacrificed at intervals following administration of choleragen in isolated ileal loops. Since previous studies in India on the infected ligated loop had stressed the finding of necrosis and epithelial desquamation, observations were also made on loops in which a drain had been emplaced to evaluate whether these changes were inherent or were a secondary response to ischemia due to increased intraluminal pressure.

A. Loops into which choleragen was injected:

Histologically the earliest changes, observed at 2 hours after injection, are congestion of the villar lacteal and a decrease in the number of mucosal goblet cells. Shortly after this there is a migration of lymphocytes and plasma cells from the lacteal across the mucosa and into the lumen. At this time there is a marked increase in the amount of nuclear debris found in the mucosa and submucosa. The entire villus appears edematous. These changes persist and increase until about sixteen hours when the intestine has become markedly dilated, the wall greatly thinned and the villi much shortened. The vessels become very congested. By twenty four hours the mucosa has become extremely disorganized and portions of it are necrotic and have sloughed.

B. Loops into which saline only was injected:

Histologically these specimens showed some edema of the villi, widening of the lacteals and an increase in the number of inflammatory cells. These specimens could, in most cases, be distinguished from those treated with choleragen by the normal number of goblet cells and the relative paucity of debris. As yet no changes in the venules have been seen where choleragen had not been used.

C. Loops into which choleragen was injected and a drain emplaced:

The principal changes consist of a very early and marked decrease in the number of goblet cells and a relative increase in the amount of nuclear debris as compared with the number of intact inflammatory cells in the mucosa and submucosa. Some edema, widening of the lacteals, and increase in the number of inflammatory cells is noted but severe congestion, thinning of the intestinal wall and necrosis is not found. In two of the specimens, and thitkeuing of smudging the wall of an occasional venule at the base of a villus is noted but this is an inconstant change in the sections examined so far.

These observations indicate: (a) that some histological changes way be brought about only by manipulation and injection of presumably physiologically inert saline solution. These are relatively slight and can be distinquished from the alterations induced by choleragen; (b) that the necrosis and sloughing, which have been observed and stressed by Indian workers studying the ligated loop, are most likely a secondary response to ischemia due to increased intraluminal pressure since they are not observed when a drain is emplaced to reduce the pressure; and (c) that the basic histologic changes brought about by injection of purified choleragen are similar to those which have been described for cholera infection in the ligated loop, in other experimental cholera systems, and in human cholera.

5, Effect of choleragen on vascular permeability

Our previous studies had indicated that purified choleragen was an extremely potent permeability factor. Subcutaneous injection of rabbits with submicrogram amounts gives rise to a delayed, sustained, erythematous, edematous induration which "fixes" intravenously administered India ink or protein-binding dyes such as pontamine sky blue (PSB) dye. Similar approaches, viz., the use of India ink or PSB failed to demonstrate vascular leakage in the small bowel of infant rabbits rendered choleraic by means of orally administered choleragen. However, by employing a somewhat smaller particle than India ink, a colloidal iron-dextran complex (Imferon). it has been possible to demonstrate that there is indeed an increase in the permeability of the villus vasculature in the choleraic infant rabbit. Imferon could be demonstrated in the excised villi of choleraic animals by means of histological stain for iron whereas positive staing was absent in control animals. The leakage was restricted to the venules of the small bowel where the Imferon could be visualized ni more-or-less discrete mottled patches. No staining was evident in the large bowel in keeping with the concept that cholera is a disease of the small bowel.

6. The effect of anti-histamines on experimental cholera

Since the above studies on the permeability effect of choleragen in the infant rabbit model indicated that the lesion primarily involved the villus venules, and resembled, in this respect, a histamine response (although the choleragen effect differs from a histamine response in the time sequence), a preliminary trial was made of the effect of some anti-histaminics on experimental cholera in infant rabbits.

Promethazine HCl (Phenergan) in a dose of 5 mg/kg BW and dipheniramine HCl (Benadryl) in a dose of 10 mg/kg BW were available for testing. Preliminary studies of gastric emptying time with barium contrast X-ray studies in the normal infant rabbit showed that Phenergan inhibited gastric emptying whereas Benadryl did not. Benadryl was therefore used in an attempt to block the choleragen induced intestinal vascular leak.

Table I summarizes the data obtained in 88 Benadryl treated and 65 control infant rabbits fed choleragen. Three observations were made over an experimental period of 21 hours: death, occurrence of over diarrhea, and the accumulation of intraintestinal fluid. There were only slight differences between the two groups in the incidence of diarrhea and in the number of animals with autopsy evidence of fluid leakage into the small intestine. However the mortality rate was greater in the untreated animals than in the Benadryl treated group. Increased survival in the latter group may be a result of slower intestinal transit time secondary to drug (see below), allowing re-absorption of fluid in the lower bower and thereby less acute fluid loss. More potent and more specific anti-histaminic drugs should be tested.

Table I

Choleragen (ug)	No Benadryl						Benadryl – treated*					
	Death		Diarrhea		Excessive fluid at autopsy		Death		Diarrhea		Excessive fluid at autopsy	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
50	17/24	71	22/24	92	23/24	96	16/41	39	34/41	83	38/41	93
25	9/12	75	10/12	83	10/12	83	8/12	67	11/12	92	12/12	100
10	10/24	42	21/24	88	23/24	96	10/30	33	18/30	60	23/30	76
4	0/5	0	3/5	60	4/5	80	0/5	0	1/5	20	1/5	20
All doses	36/65	54	56/65	86	60/65	92	34/88	39	64/88	73	74/88	84

* Animals were given 1 mg of benadryl I.P. 1/2 before, simultaneously, and 1/2 h after feeding of choleragen.

Determination of G-I Transit time in normal and benadryl-treated infant rabbits. Carmine red dye, a non absorbable marker, was used as an indicator of transit time. 5 mg of dye was added to 1 ml of phosphate buffer and fed to infant rabbits. One group of animals was given 1 mg of benadryl intraperitoneally twice, 1/2 h before and after feeding of dye, another group was fed dye only. All animals were put in separate partitions of a cage and observed for the appearance of red stool every 15 (and later on 30) minutes. The results are shown below.

Time (h)	Number and percentage of animals excreted dye			
	Control		Benadryl – treated	
	No.	%	No.	%
at – 6	0	0	0	0
6 – 7	3	10.3	0	0
8 – 9	5	17.2	1	3
11 – 12	9	31.0	3	10.8
13 – 20	20	69.0	13	46.8
21 – 22	25	86.5	16	57.6
at – 24	28	96.6	23	82.8
Total No.	29	100.0	28	100.0

Benadryl inhibits intestinal transit time in infant rabbits in a dose of 10 mg per kg body weight. This non specific effect may be the mechanism whereby Benadryl appears to enhance survival in experimental cholera.

7. Production of intestinal immunity by parenteral vaccination with choleragen

Adult rabbits were inoculated simultaneously at multiple sites (subcutaneous, intramuscular, intraperitoneal and in foot pads) with 5 mg of choleragen in complete Freund's adjuvant. Three weeks later, the animals were bled for antibody determinations. At that time choleragen was assayed in them by intradermal inoculation and by the loop technique described above. In addition, one loop was inoculated with 10^8 vibrios of strain 569 B. The same procedures were performed simultaneously in control animals.

In the intradermal tests, the smallest doses of choleragen that produced positive reaction in the control group of animals were 0.005-0.01 ug, while in the immunized group they were 1.25-2.5 ug, 250 times higher. The responses to intraintestinal challenge are summarized in Table II. The results demonstrate conclusively that under these conditions it is possible to produce relatively strong immunity both to choleragen, intradermally and intra-intestinally, as well as to the intra-intestinal inoculation of a rather massive challenge of cholera vibrios. Serum from each of the immunized animals, except #5, was demonstrated to contain precipitating antibody against choleragen and to neutralize its choleragenic effect when serum and cholerage were mixed prior to feeding infant rabbits. Serum from rabbit #5 had a lower content of choleragen antibody and did not completely neutralize choleragen fed to infant rabbits. When pooled together and given intraperitoneally to infant rabbits 18 hours prior to oral choleragen, these sera offered some protection, although not absolute, against experimental cholera.

Table II

Effect of Parenteral Immunization* with Choleragen on Resistance to Intestinal and Intradermal Challenge

Challenge	Control Rabbits				Immunized Rabbits					
	1	2	3	4	5	6	7	8	9	10
Intra-intestinal										
10^8 V. cholerae	3.3**	7.0	5.8	6.7	2.8	0	2.8	0	0	0
Choleragen										
50 ug	—	—	—	—	2.6	0	1.0	5.3	0	0
10 ug	5.1	7.0	5.6	6.6	0	0	2.3	0	0	0
2 ug	0	0.8	4.0	5.0	0	0	0	0	0	0
0.4 ug	0	0	0	4.0	2.5	0	0	0	0	0
0.08 ug	0	0	0	0	—	—	—	—	—	—
Intradermal Choleragen	0.005#	0.01	0.02	0.005	1.25	2.5	> 10.0	2.5	1.25	5.0

* Immunized rabbits were inoculated with 5 mg of choleragen in Freund's complete adjuvant in multiple sites 3 weeks prior to challenge.

** ml of fluid per inch of intestinal loop, 18 hours after challenge.

\# Smallest dose of choleragen producing reaction, 18 hours after inoculation. Rabbits 9 and 10 were pre-tested, prior to immunization. The minimal skin reactive doses at that time were 0.01 and 0.15 ug, respectively.

8. Production of experimental cholera in Thiry-Vella loops in adult rabbits.

Preliminary experiments have suggested the feasibility of producing experimental cholera in modified Thiry-Vella fistulas in adult rabbits. Open lengths of ileum, ranging from 15 to 25 cm, were isolated, with blood supply intact, and anchored at both ends to holes in the abdominal wall. The stomata could be occluded with Foley catheters to enable administration of choleragen and collection of intestinal fluids. The first two animals were administered 50 ug of choleragen, in 5 ml, in the isolated loops. After 1 hour the loops were drained and fluids were collected overnight. During this period approximately 40 ml of "rice water" fluid was excreted. Studies on the electrolyte composition of the fluids are in progress. This model offers great possibilities for controlled studies on the development of immunity and on electrolyte balance.

9. Choleragen in the canine model

On the occasion of the NIH Cholera Advisory Committee Wor- [illegible] holera which was held at Johns Hopkins University School of Medicine, December 6-8, the coor [illegible] laboration with Dr. C.C.J. Carpenter and Dr. Bradley Sack, administered purified cholerag [illegible] rel dog. The dog, which weighed 14 kilograms, was given 80 mg of choleragen by intraduoden [illegible] Fluid produced in the intestinal tract was drained by means of a catheter placed in the termin[illegible] ileum. Over a 35 hour period following administration of choleragen, the dog lost over 1 liter of clear, rice-watery, non-hemorrhagic fluid (from the intestinal drain and in vomitus) confirming the fact that the choleragen preparation contains the toxin responsible for fluid and electrolyte loss in (canine) cholera. Previous trials, in which amounts of less than 10 mg were given to dogs in the same manner, suggest that the dog is somewhat more refractory to choleragen than the infant rabbit or man.

10. An attempt at improving conventional cholera vaccines

Almost universally, current conventional cholera vaccines are composed of killed vibrios derived from the (U.S.) NIH Inaba and Ogawa serotype reference strains although there is no evidence that these strains are superior to any others. The results of the recent field trials of cholera vaccines have indicated that some presently available vaccines confer some limited degree of immunity against cholera for a short duration. Obviously some improvement is needed. Accordingly, it was decided to attempt to improve conventional vaccines by selection of strains of higher antigenicity

A test was devised to evaluate the antigenicity of various strains of cholera vibrios in the rabbit. The test was based on the observation that the agglutinin response in rabbits inoculated with a single vaccine dose is directly proportional to the amount of vaccine used. Accordingly, a comparison was made of the agglutinin response of rabbits, 4 per group, inoculated with single doses of monovalent vaccines composed of strains representing each of the kinds of choleragenic vibrios; El Tor Inaba and Ogawa and classical Inaba and Ogawa. The rabbit sera were each tested first against agglutinating antigens prepared from the NIH reference Inaba and Ogawa strains. On the basis of these results, the "best" and the "worst" strains in each category (of approximately 10 strains per group) were selected and their sera were titrated individually against a battery of agglutinating antigens (8 Inaba and 7 Ogawa types). The geometric mean titers resulting in these assays are abridged in Table II. Comparison of the results with paired "good" and "bad" strains revealed the differences to be statistically significant (95% confidence) in all but the comparison between strains V. cholerae 569 B and IDH 59. Based upon these results, two quadrivalent vaccines, a "good" one and a "bad" one, were composed of the odd and even numbered strains (Table III), respectively. These have passed rigid animal and laboratory tests for sterility, safety and toxicity. The quadrivalent vaccines were compared by a dose-response antigenicity assay. The results of this test, in which graded single doses of the vaccines were administered to groups of rabbits which were bled for agglutinating and vibriocidal.

Table III

Comparison of geometric mean agglutinin titers* elicited
by "good" and "bad" vaccine strains

Strain Number	Designation	Geometric Mean Titer**	95% C.L.
1	V. chol. Inaba 569 B	760.9	475 - 1219
2	" " IDH 59	603.8	369 - 784
3	El Tor Inaba BRL 7738	1070.0	810 - 1430
4	" " HP-51.1	356.7	270 - 471
5	V. chol. Ogawa 12RX1	1312.0	1047 - 1644
6	" " VN Dalat	320.0	249 - 412
7	El Tor Ogawa VN 258	689.5	560 - 848
8	" " Teheran 816.0	362.1	256 - 489

* Based upon titrations of 4 sera per strain against a battery of agglutinating antigens (8 Inaba types and 7 Ogawa types)

** Homologous antigenic type titers only.

antibody determinations after two weeks, indicated that the test had all the attributes of a valid bioassay and that the response to the two vaccines was significantly different* by a factor which represents their relative potency in this test. With regard to the Inaba agglutinin response, Vaccine A is 13.48 times more potent than Vaccine B (95% C.L. 5.415 and 33.59). With regard to the Ogawa response, Vaccine A is 8.990 times more potent (95% C.L. 4.197 and 23.15).

The two vaccines were assayed in the standard mouse potency test by Dr. John Feeley, Division of Biologics Standards, National Institutes of Health. Interestingly, in those assays, the two vaccines did not differ significantly and were found to be equivalent to reference standard potency preparations.

It is hoped to project these studies to small groups of human volunteers who will be given single doses of diluted vaccines to determine if the rabbit assay predicts the human serological response.

* We are indebted to Col. S. Vivona, Director, US Army Medical Component, SEATO Medical Research Laboratory for his assistance in the statistical interpretation of these data.

Summary

1. Choleragen is produced in a completely defined medium supplemented with 16 L-amino acids. Purified choleragen has been isolated from crude cholera culture filtrates produced in rather large volumes at WRAIR. A non-choleragen elaborating strain did not inactivate pre-formed choleragen.

2. DEAE cellulose may be useful in further purification of choleragen. Choleragen has been identified by sucrose density gradient centrifugation as having a sedimentation coefficient approximating 6.7 S. The technique of preparative disc electrophoresis is being investigated for possible application to the ultimate purification of choleragen.

3. Choleragen is elaborated in vivo in ligated loops in adult rabbits by some strains of cholera vibrios during the phase of their logarithmic increase in numbers during infection. It can be assayed by skin reactivity and by precipitation with antibody in Ouchterlony tests. Some strains which fail to produce immunologically detectable levels of choleragen in vivo may produce the small amounts which are required to initiate the positive loop.

4. Histological observations on the development of the positive loop in response to choleragen indicate that the necrosis and epithelial detachment are a consequence of ischemia due to increased intraluminal pressure.

5. Choleragen has been shown to alter vascular permeability in the small bowel of infant rabbits. The vessels involved are primarily the venules.

6. Some anti-histaminics may exert a slight non-specific apparent pr[illegible] effect on experimental cholera in infant rabbits.

7. Immunization with purified choleragen in Freund's adjuvant gave rise to a strong degree of intestinal immunity to challenge with choleragen or with live vibrios in adult rabbits.

8. The Thiry-Vella fistula, in adult rabbits, offers great possibilities for controlled studies on the development of immunity and electrolyte balance in cholera.

9. Choleragen also causes a cholera-like syndrome in the canine model.

10. A new assay has been developed for selecting highly antigenic strains for inclusion in conventional cholera vaccines. Vaccines composed on the basis of this test were found to differ significantly in their relative potencies in the antigenicity test but not in the mouse protection test. It is hoped to project these studies to small groups of human volunteers to determine whether this laboratory test predicts response in man.

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SEATO MEDICAL RESEARCH STUDY ON DIARRHEAL DISEASES

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Period of Report: 1 April 1966 - 31 March 1967

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General:

This study includes projects concerned with the effects of antibiotics on gut function and structure (including histochemistry), the prevalence of lactose intolerance in Thais and Americans living in Thailand, the effect of daily lactose feeding on lactose metabolism in Thais, the effects of acute diarrhea on gut function and structure in Thais, the effect of ceroid pigment deposition on intestinal function in northeast Thailand, and the quantitative changes in the fecal flora of healthy and diarrheic persons. The following projects were started: a long-term study of the effect of a tropical environment on gut morphology and function in Peace Corps volunteers and in Special Forces personnel, and in the latter, the effect of daily folic acid; a study of the immunological defense mechanisms of the Thai intestine in both normal subjects and in those with acute diarrhea; and a quantitative study of the bacterial flora of upper gastrointestinal fluids aspirated from persons with and without diarrhea.

In conjection with the department of medicine of the Royal Thai Army Hospital, several well-documented cases of malabsorption due to tropical sprue were thoroughly studied and added much to our knowledge of this disease in Thailand. Fortunately, tropical sprue appears to be quite rare in the Bangkok area, and to our knowledge, has never been documented in an American living anywhere in Thailand.

Study Report | Bacteriologic Survey of Stools from Patients with Acute Diarrhea

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The objectives of this study were to determine the types, frequency of accurrence, and pattern of antibiotic sensivity of salmonellae and shigellae among patients with acute or chronic diarrhea. Stools were studied in an effort to determine the incidence of other Enterobacteriaceae and their relationships to this disease.

This study included specimens from inpatients and outpatients of both sexes from hospitals throughout Thailand. Most of the specimens were collected during the acute phase of the disease from patients hospitalized with diarrhea. During the first 11 months of this reporting period the laboratory procedure was as follows: In the Bangkok area either fecal specimen or three rectal swabs, moistened in alkaline peptone broth, were obtained from each patient. Two of the swabs were placed in enrichment broths (alkaline peptone, selenite-F) and the third streaked directly onto SS and MC agar plates. The alkaline peptone broth was subcultured to alkaline lauryl sulfate tellurite agar for isolation of vibrios. After overnight incubation at 37 C, the selenite-F broth was subcultured on SS and DC plates. Specimens from outside Bangkok were submitted in a holding medium designed for the transport of enteric bacteria. Upon arrival at the laboratory, plates of SS and MC were streaked and tubes of selenite-F and alkaline peptone broth inculated. The enrichment broths were subcultured as outline above. All plates were examined after 24 and 48 hours incubation. Lactose-negative colonies were transferred to Kligler's iron agar slants and subsequently to a battery of media to determine patterns of biochemical activity. Those isolates showing biochemical patterns typical of salmonellae, shigellae, or vibrios were definitively identified in accordance with the serological methods described by Edwards and Ewing.

During the last month, the procedures for processing stool specimens were modified to incorporate a technique for the selection of suspicious colonies from initial isolation media by using transmitted oblique light and a stereosopic dissecting microscope. A more rapid method for determining carbohydrate fermentation patterns is also being evaluated. These techniques are described more fully below.

During this reporting period a total of 2935 specimens were examined from 1786 (1306 Thai, 480 caucasian) patiants with acute diarrhea. Recognized diarrheal agents were isolated from 21% of these specimens. Approximately 15.9% of the specimens yielded salmonellae, 5.3% shigellae and 2[illegible].6% paracolons (Table I). These data are consistant with the recovery rates which have been remarkably stable during the preceeding four years. Enteropathogenic Escherichia coli (E.E. coli) were recovered from 7.8% of 2231 (2062 Thai, 169 caucasian) isolates from children under 6 years of age (Table IV) E.E. coli were found

with twice the frequency among Thais as Caucasian (8,1% Thai, 4.1% Caucasian); however, a larger number of caucasian children must be studied to determine the significance of these data. The finding of enteropathogenic serotypes in infants and small children suffering from acute diarrhea emphasizes the role of these organisms as a pathogen for this age group.

The data in Table II show 18 species to be represented among the 468 salmonellae isolates. S. paratyphi B was the predominating organism and accounted for 58.5% (274/468) of the salmonellae isolates. S. derby accounted for 19.0% with approximately equal numbers of S. weltevreden, S. anatum, and S. montevideo comprising another 11.7%. Isolates of the salmonellae species most frequently recovered, were found to be evenly distributed throughout the year. One exception was noted when an epidemic caused by S. derby resulted in 69 isolates during a one month period. The isolates of S. montevideo decreased for the second consecutive year. In 1964, S. montevideo was the predominant enteropathogen and accounted for 51% of the salmonellae isolates. Recovery percentages dropped to 11.2% in 1965 and to low of 4.0% during this reporting period. The recovery rates for salmonellae (5.8%) and shigellae (5.2%) were approximately equal among the caucasians in this study, whereas salmonellae were 3 times as prevalent as shigellae among the Thai nationals. The significance of these data is unknown at this time, and will require additional investigation.

There were 11 species among the 155 shigellae isolates obtained during this reporting period (Table III). The most frequently encountered species were: Sh. flexneri 3, 40.6%; Sh. flexneri 2, 22.6%; Sh. sonnei form 1, 16.8%; and Sh. dysenteriae 1, 7.7%. Isolations of the various species of shigellae were well distributed throughout the year with no single epidemic accounting for an abnormal frequency for any given species.

Agglutinating and non-agglutinating vibrios were rarely isolated in the Bangkok area. Outbreaks of cholera requiring the assistance of personnel from this laboratory will be reported under the Study Report on Cholera.

Antibiotic sensitivities were determined for 406 enteric pathogens in an effort to maintain a continuing surveillance of the drug susceptibilities of these organisms in Thailand. Six antibiotics (tetracycline, colistin, kanamycin sulfate, chloramphenicol, neomycin sulfate, nalidixic acid) were used in a tube dilution procedure, and the results obtained with 159 salmonellae, 155 shigellae, and 92 E.E. coli isolates are shown in Tables V through X. These data show nalidixic acid and colistin to be uniformily effective in vitro, with the organisms exhibiting varying degrees of resistance to the other drugs. Determinations are still in progress, therefore, a detailed comparison with the studies conducted by Dr. Noyes in 1963-1964 has not been made. There is, however, an apparent increase in resistance, and examples of this shift in drug susceptibility can best be illustrated by the results obtained with the predominating Salmonella and Shigella isolates. These data show 30 of 40 Sh. flexneri 3 isolates to be resistant to < 100 mcg of tetracycline, whereas Noyes found only 2 of 17 resistant at this level. Twenty of 21 S. paratyphi B are resistant to < 100 mcg of either tetracycline or neomycin sulfate. Two years ago 7 of 10 and 6 of 10 S. parathphi B isolates were resistant to these levels, respectively.

In July, a survey for enteric pathogens among the children and attendants at the Central Preventorium for Children (Nondhaburi Province) was conducted to provide baseline information prior to conducting a proposed longitudinal study in this groop. Two hundred and forty seven stool specimens obtained from 227 persons (152 children under 6 years of age and 75 adults) resulted in the isolation of 27 strains of salmonellae (11%), 4 strains of shigellae (1.7%) and 39 strains of E.E. coli (16%). Additional breakdown of the results showing the recovery of pathogens from asymptomatic versus individuals with diarrhea is presented in Tables XI and XII.

During the November 1966 visit of Dr. Samuel Formal, Chief Department of Applied Immunology MRAIR, arrangements were made with the Special Forces unit in Thailand to study the diarrheas among their

personnel. Specimens from acute diarrhea were to be collected in holding medium and sent to SMRL for culture. An information sheet was to accompany each specimen. Unfortunately, it has been impossible to obtain fecal specimens from this group, and hope for this aspect of the study has been abandoned. Captain Blaydow, the S.F. surgeon, has submitted 109 questionnaires which provide some information on the amount of diarrhea occurring in this group of approximately 300 men. These men are assigned in small groups to many areas in Thailand, and not all segments have been participating on a regular basis. However, by making a few estimates the following approximations were obtained. There has been approximately 4905 man days of diarrhea out of a total of 28,800 man days incountry. The average time of onset of diarrhea was 7.6 weeks after arrival, with a 4.5 day duration of illness. The average number of stools per day was 6, with 24% complaining of fever and 41% having cramps. The type of unit being studied, and the fact that only those men reporting to the dispensary with diarrhea are included in the statistics, tends to make these data obvious underestimations of the amount of diarrhea occurring in this group. These data do provide some information on the amount of diarrhea in these troops, and is perhaps the closest approximation that can be obtained.

In May of 1966, a survey of bacterial enteropathogens and parasites was conducted in Nakornpanom (northeast) Thailand. The recovery of bacterial pathogens among adults and children with and without diarrhea is presented in Table XIII. The intestinal parasites are listed in Table XIV.

A study was undertaken in August of 1966 to evaluate a rapid technique for the detection and identification of enteropathogenic organisms. The technique was reported by Sanders, A.C. et al (Appl. Microbiol. 5: 1957). It was designed for use in diarrheal epidemics, but had not been evaluated using freshly isolated organisms or under field conditions. The rapid technique differs from the classical methodology only after initial isolation, when a 2 hour incubation in "booster broth" is used to screen lactose from non-lactose fermenting organisms. The booster broth serves as a medium for the determination of indol production, urease activity and motility as well as inoculum for pour plates and serological typing. The carbohydrate fermentation patterns of non-lactose fermenting organisms are determined by preparing a pour plate, and placing 4 paper discs saturated with dextrose, lactose, sucrose and mannitol on the surface of the agar. The production of acid and H_2S is detectable, around the appropriate carbohydrate discs, after 6 hours incubation, and further identification is then possible by serologic typing of the organism. Using this technique it is theoretically possible to isolate and identify an enteropathogen within a 2 day period.

Initial evaluation of the rapid technique consisted of a direct comparison with the classic methodology routinely used at SMRL. In order to process the same colony through both methods it was necessary to pick each colony from the isolation medium to a Kligler's iron agar slant to provide sufficient inoculum for use in both identification schemes. Eighty five suspected colonies were processed by both methods and 16 strains of salmonellae and 2 strains of shigellae detected. Results were identical with both methods.

One hundred forty one stool specimens (34 adult, 107 children) from Praputhabath Provincial Hospital provided additional opportunity to evaluate the Sander's technique. Recognized pathogens were isolated from 28.4% of these patients, and the distribution of species among the 13 salmonellae, 13 shigellae and 14 E.E. coli is shown in Table XV. All 13 of the shigellae species were isolated during a small outbreak of shigellosis, which explains the unexpected recovery of equal numbers of shigellae and salmonellae from this central Thailand community. This method appears to be a simple and reliable technique for the rapid identification of enteropathogens. The opportunity has not arisen to conduct field tests during a large epidemic, however, it should prove to be an effective technique for handling large numbers of specimens. Sander's method combined with the use of transmitted oblique illumination (reported below) is currently being evaluated for use as a routine method in the enteric bacteriology section.

Dr. Richard A. Finkelstein with the assistance of Miss Kannikar Punyashthiti investigated the feasibility of using transmitted oblique illumination to reveal colonial differences which might be used for the recognition of enteropathogens. A report of their investigation is included.

Study Report A "New" Approach to Diagnostic Enteric Bacteriology

Principal Investigator: Richard A. Finkelstein, Ph.D.

Associate Investigator: Kannikar Punyashthiti, M.Sc.

Conventional methods of bacteriological diagnosis of enteric disease suffer from the severe shortcoming that results are not usually available during the acute illness of the patient. Accordingly, it would be highly desirable to develop more rapid methods for recognition of enteric pathogens. We, and others, have observed that transmitted oblique illumination reveals differences in color, refractility and internal structure of bacterial colonies on transparent media which are not apparent with other forms of illumination. These colonial differences are heritable, and the colonial morphology, under defined conditions, is characteristic for different bacterial genera or species. With the use of an ordinary stereoscopic dissecting microscope, the ability of the observer to detect colonial differences is enhanced even further.

With some experience, the technician can be trained to recognize these differences and to associate particular colonial appearances with particular genera and species. Simply by observation, then, he is enabled to make educated guesses regarding the identity of the bacteria present which can, and must, then be confirmed or refuted by selection of appropriate rapid tests. For example, suspecting a Salmonella from the colonial appearance, the technician could immediately perform slide agglutination tests for both serological grouping and typing.

With experience, the accuracy of these "guesses" should approach or exceed 90%. This results in great savings of time, materials, and manipulative steps without sacrifice of accuracy and with even some increase in sensitivity over conventional procedures. The technique allows the laboratory to establish a diagnosis in the case of enteric disease before the patient is either recovered or dead.

The system is easy to set up in any laboratory. One needs only a microscope lamp, a mirror, and a low power dissecting microscope. The mirror is placed flat on the table, in front of the microscope, and the light is directed into the mirror and reflected by it upwards, through the Petri dish on the microscope stage, at an angle of approximately 40°. It should be adjusted, by the observer, for maximum contrast.

We have prepared a series of color photographs of colonies of the common enteric pathogens and commensal organisms on MacConkey's agar which serves as a "rogues gallery" for reference while using the technique and as an aid in training.

A comparison between the proposed technique and conventional methodology has been made on a series of 68 consecutive enteric specimens. The conventional method employed a battery of selective and differential isolation media, including MacConkey's agar, and enrichment procedures. Only the MacConkey's plate was used in the "new" technique. The conventional methods yielded a total of two salmonellae strains and five shigellae. Nine salmonellae and the same five shigellae were identified by the proposed technique. One Salmonella isolate, which was obtained by the conventional technique only after selective enrichment, was missed using the "new" approach.

Summary:

Bacterial pathogens were recovered from approximately 21% of 2935 stool specimens from individuals with acute diarrhea. Salmonellae were isolated from 15.9%; shigellae, 5.3%, and paracolons, 26.6%. The most frequently isolated enteropathogen was S. paratyphi B followed by S. derby, Sh. flexneri

3 and *Sh. flexneri* 2. *Enteropathogenic E. coli* were recovered from 7.8% of the stool specimens from children under 6 years of age. Antibiotic susceptibilities for more than 400 isolates of enteric pathogens, to 6 antibiotics, were determined using a tube dilution technique. All isolates were uniformily susceptible, in vitro, to colistin and naladixic acid with varying resistance to tetracycline, chloramphenicol, neomycin sulfate and kanamycin sulfate. A survey being conducted in a Special Forces unit in Thailand indicates diarrhea not to be a major cause of morbidity in this group. The average time for onset of diarrhea was 7.6 weeks after arrival in country, with a 4.5 day duration of illness. A technique for colonial recognition of enteric organisms using oblique transmitted light and a stereoscopic dissecting microscope was evaluated as a practical procedure for use in the routine enteric bacteriology laboratory. A rapid technique for determining the carbohydrate fermentation patterns of enteric pathogens was also evaluated, and found to be reliable. A combination of these two techniques is currently being evaluated. Preliminary indications are that identification of enteropathogens can be made more readily than when classical procedures are employed.

Title: Quantitative changes in Bacterial Flora of the Gastrointestinal tract during Diarrheal Diseases

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The objective of this study is to quantitate the gastrointestinal bacterial flora of persons with and without diarrhea, and to determine differences, if any, between these two groups. Additional studies include the influence on intestinal flora of antimicrobial agents administered therapeutically to diarrhea patients or experimentally to non-diarrhea controls. Quantitative bacterial counts were obtained in 50 normal Thai adults before and after prolonged supplementary lactose in their diets. An investigation was also initiated to determine the incidence of antibiotic producing E. coli in the stools of children with and without clinical diarrhea.

Studies designed to quantitate the fecal bacterial flora were teminated last year, and similar studies utilizing intestinal juices were conducted during this reporting period.

Specimens were obtained by aspirating intestinal fluid through orally administered tubes. The total time lapse between collection of specimens and completion of processing was usually less than 1 hour for anaerobic incubation and less than 2 hours for aerobic incubation. Plate counts, on serial dilutions from 10^{2} to 10^{10}, were carried out on the 9 media listed in Table XVI. In those instances when the same species of organism grew on more than one medium, the medium with the highest count was used. Routine bacteriological, biochemical, and serological procedures were used for final identification of organisms.

During the period of this report, duodeno-jejunal or jejunal fluid was obtained from 40 normal adult Thais, 20 normal children and 5 adults and 2 children with diarrhea. Ninteen of the 40 specimens from normal adults had at least 1 organism with counts greater than 10[illegible]/ml. The most frequently encountered species were neisseria, coliforms, anaerobic streptococci, gram positive cocci, and pseudomonas. Four of the 5 intestinal fluids from adults with diarrhea had significant growth (more than 10[illegible] org/ml) of gram positive cocci, anaerobic streptococci or coliforms. Significant numbers of Pseudomonas spp. were also obtained from 2 samples. The total counts of most organisms from the upper gastrointestinal tract of both adults and children were from 10[illegible] to 10^{6}/ml of aspirated fluid.

Six of the 20 jejunal samples from normal children had at least 1 organism in concentrations greater than 10^{3}/ml. All 3 specimens (2 duodeno jejunal and 1 jejunal fluid) obtained from 2 children with diarrhea showed significant numbers of E. coli. The range of total bacterial counts in aspirated fluid from the upper intestinal tract of patients with diarrhea were from 10[illegible] to 10[illegible], but bacterial counts were slightly higher than found in "normal" persons.

Tissue from 28 biopsys of the jejunum were compared with 20 fluid aspirates collected at the same time. Only 4 biopsy specimens (28%) had 10^{3} orgs/gm of tissue, whereas 12 fluids (60%) showed 10[illegible] orgs/ml.

Four presumably normal infants were given kaomycin (neomycin plus kaolin) to determine the effect of this drug on their fecal flora. Unfortunately, all four children had to be dropped from the protocol for various reasons. One child developed multiple abscesses on the neck and she was given chloromycetin therapy. S. derby was isolated from the stool of another child who was dropped from the study, but did not develop clinical diarrhea. The remaining 2 children both became ill with diarrhea. E.E. coli, 0119:B11, and Sh. sonnei from 1 (10[illegible] orgs/gm of feces) were isolated from one child and S. anatum from the other. No additional drug studies were undertaken in children, however, quantitative studies on intestinal fluids from 7 normal adult Thais were studied before and after 4 grams of neomycin administered within a 24 hour period. Seventeen upper intestinal aspirates were obtained before administration of antibiotic and eleven after. A summary of the results is persented in Table XX. Eighteen intestinal fluid samples were obtained from 13 adult Thais after administration of tetracycline (5 subjects, 7 specimens), neomycin (5 subjects, 7 specimens), chloroquine (1 specimen), folic acid (1 specimen) and prednisone (1 subject, 2 specimens). No significant changes were noted either during or after the administration of each drug, with the exception of tetracycline when the organisms appeared to increase quantitatively in 2 of the 5 individuals.

Fifty normal Thai marines stationed at Sattahip were studied prior to and after the subjects were given 50 grams of lactose solution per day for one month. The objective of this study was to obtain baseline information on the bacterial flora of the small intestine in normal Thais, and the subsequent changes in fecal and intestinal flora after prolonged supplementary lactose in the diet. In conjunction with a lactose tolerance and intestinal biopsy study, upper small intestinal fluid was obtained by aspiration through a second tube which was inserted together with the Crosby Kugler capsule. Stool specimens were collected at the beginning and termination of the experiment for quantitative bacterial studies. Due to limited field laboratory facilities and time limitations, it was impossible to do complete quantitative studies on the stool specimens. Therefore, only the numbers of lactobacilli were quantitatively examined. Forty two pre-lactose and 47 post-lactose stool specimens were studied. The mean difference in total lactobacilli before and after lactose administration indicates a slight increase in lactobacilli after one month of lactose. However. examination of the data is not complete, and the range of the total bacterial counts was so great (10[illegible] - 10[illegible]), both pre and post lactose, that statistically significant differences in flora may be impossible to demonstrate.

The examination of intestinal fluid was conducted by preparing serial dilutions of the aspirated specimen and plating, by surface inoculation, onto the 9 media listed in Table XVI. The method was designed to detect organisms in concentrations as low as 100 per ml. Forty three controls (pre-lactose intestinal fluids) were quantitated. The results of the pre-lactose fluids were; no growth from 16% 10[illegible] 10[illegible] organisms from 42% and an equal number (42%) with counts greater than 10[illegible]/ml. The distribution of counts for the 48 post-lactose fluids were 21%, 48%, and 31%, respectively. The percentage recovery for each group of organisms is presented in Tables XVII, XVIII and XIX. The predominating organisms were streptococci (aerobic and anaerobic), enterococci and Neisseria spp. Other organisms were found only in lower numbers and less frequently. The significance of the large numbers of streptococci and Neisseria spp. in the upper intestinal fluid is questionable, since there are indications that these organisms may have been introduced into the specimens from higher in the gestrointestinal tract. Our previous experience with fluid obtained through the intestinal wall, at autopsy, did not reveal the presence of Neisseria spp. Similar studies described in the literature do not report the presence of Neisseria spp. when specimens were collected either during surgery or at autopsy. It was also noted in this study that when Neisseria spp. were found, the numbers of alpha streptococci also increased. For these reasons, it is entirely possible that at least some of the neisseriae and alpha streptococci were mechanically introduced, during the sampling procedure, from higher in the gastrointestinal tract. It should be noted from Table XVII that neither Clostridium nor Bacteroides spp. were recovered from any of the intestinal fluid specimens. The analysis of these data is not complete, however, the general impression is that the recovery percentages of coliform organisms was higher and the gram positive organisms lower after the administration of lactose.

A preliminary investigation was undertaken to determine the presence and prevalence of antibiotic producing E. coli in the stools of children with and without diarrhea. The initial phase consisted of a search

for a susceptible organism to serve as an indicator for the detection of antibiotic producing E. coli. Eighty strains of shigellae were checked for susceptibility to 29 antibiotic-producing E. coli, and the organisms selected as the indicator was a strain of Sh. sonnei form 1 which had been isolated from an American serviceman stationed in Thailand. Optimal conditions for conducting the assays were determined by studying the effects of quantitative changes in concentration of both the indicator shigellae strain and the E. coli inoculum, the effects of lapsed time between seeding the two organisms, and the influence of incubation time on the zone of inhibition. The following standard procedure was adopted. Samples of fecal material were obtained either by rectal swabs or stool specimens. They were streaked on MacConkey's and Salmonella Shigella plates for isolation, and at least 10 colonies (5 from each medium) of suspected E. coli were transferred into Kligler's iron agar slants for further biochemical confirmation. E. coli strains were stocked for subsequent testing against the standard indicator Shigella strain. Enteropathogens isolated from diarrhea patients were also checked for susceptibility to strains of concomitantly occuring antagonistic E. coli. The assay for antibiotic producing E. coli was carried out in the following manner. Sixteen to 20 hour cultures of the susceptible strain of Sh. sonnei were diluted with sterile saline to approximately $10^5 - 10^6$ organisms per ml. The suspension was then flooded onto the surface of proteose # 3 agar plates, the excess drained, and the plates dried by incubation at 37°C for 30 minutes. Circular areas 7 to 10 mm. in diameter were inoculated with undiluted E. coli, permitting the assay of 8 strains on each plate. After 24 hours incubation, at 37°C, the zones of inhibition around the E. coli inoculum were measured and recorded as; 0 or inactive (no inhibition), + or slightly active (up to 2 mm inhibition), + + or moderately active (greater than 2 mm inhibition).

The subjects for this study were all children under 2 years of age. Two groups were studied. Specimens from a non-diarrhea group were obtained from children in a private nursery, and specimens from children with diarrhea from patients admitted to the Royal Thai Army Hospital. A total of 1238 strains of E. coli were isolated from 111 specimens collected from 29 children. All were screened for inhibitory activity. In these limited data, the incidence of antibiotic-producing strains of E. coli was highest among the diarrhea patients with shigellae, or shigellae in combination with salmonellae. Patients with salmonellae, or salmonellae in combination with other pathogens, appeared to harbour fewer but more active antibiotic-producing E. coli (Table XXI). One asymptomatic child was found to be carrying both Salmonella newport and S. weltevreden. Thirty nine strains of E. coli from 3 specimens were assayed and 7 strains were found to be active. Five fo the 7 strains were highly antagonistic to the indicator strain, but none were active against the patient's own salmonellae. No active strains were detected after the patient was put on chloromycetin therapy nor during an episode of diarrhea of unknown etiology which developed after discontinuation of the drug.

Attempts were made to follow changes, if any, in the numbers of antibiotic-producing E. coli during the course of the diarrheal disease. These data are presented in Tables XXII and XXIII.

Eight shigellae isolates from patients with diarrhea were checked for susceptibility to their own strains of antibiotic producing E. coli. None of 3 strains of Sh. flexneri 3 were susceptible; however, 3 strains of Sh. sonnei form I, and a single isolate of Sh. dysenteriae 1 were inhibited by their own E. coli strains. Salmonellae strains were uniformily resistant to the inhibitory effects of E. coli, in contrast to the susceptibility of all Enteropathogenic E. coli isolated from children with diarrhea. An interesting observation was noted with isolates from a patient with diarrhea caused by Sh. boydii 6. The E. coli strains from this patient were not antagonistic when assayed with the standard Sh. sonnei indicator strain; however, they were inhibitory for the patient's pathogen, Sh. boydii 6. This finding combined with the low percentage of antibiotic producing E. coli found in this study, when compared with similar studies conducted in the U.S. (Tables XXIV and XXV), indicate a more sensitive indicator strain should be sought.

An investigation was initiated to determine the frequency with which various serotypes of Enteropathogenic E. coli produce antibiotics. Fifty three strains of E. E. coli, isolated from 53 children with

diarrhea, were assayed for activity using the standard Sh. sonnei strain. Only 3 strains showed a slight degree of activity. One hundred and four strains of antibiotic producing E. coli, isolated from all sources, were then checked for the presence of E. E. coli antigens, and all strains were found to be negative. These data indicate E. E. coli to be very poor antibiotic producers, but uniformly susceptible to the antibiotics produced by other E. coli.

Summary: Fluids aspirated from the upper gastrointestinal tract of children and adults, both "normal" and during diarrheal disease, contained significant numbers of bacteria (10^4 orgs/ml). The flora was predominantly gram positive cocci, fecal streptococci, lactobacilli and yeasts. The total counts of most organisms ranged from 10^2 – 10^6 per ml of aspirated fluid. The bacterial counts were slightly higher in diarrhea patients. No significant numbers of bacteria were detected from intestinal biopsy tissue. The administration of neomycin sulfate, chloroquine, folic acid, or prednisone appear to have no influence on bacterial flora of intestinal fluids. In contrast, there was an increase in bacterial flora in 2 of 5 persons receiving experimentally administered tetracycline. The bacterial flora of 42% of the upper intestinal fluid samples, from normal Thai adults, contained at least 10^3 organisms per ml. After 50 grams of lactose solution per day for 30 days, the quantitative bacteriology of upper intestinal fluid was much the same as initial control specimens. Two groups of children, with and without diarrhea, were surveyed for the prevalence of antibiotic producing E. coli in their stools. 1238 strains of E. coli were screened for antibiotic production. The incidence of antagonistic E. coli was highest among patients from whom shigellae, or shigellae and salmonellae were both isolated. The lowest incidence was among diarrhea patients from whom salmonellae were recovered. Whenever possible the quantitative changes in antibiotic producing E. coli were followed throughout the course of the diarrhea. The incidence of antagonistic E. coli was lower in this study than previously found in the U.S. Only 3 of 53 Enteropathogenic E. coli strains exhibited inhibitory activity. Enteropathogens isolated from diarrhea patients varied in their susceptibility to the inhibitory activity of concomitantly occurring E. coli strains. E. E. coli were uniformly susceptible as were Sh. sonnei form I, Sh. boydii 6 and Sh. dysenteriae 1. All salmonellae and the 3 isolates of Sh. flexneri 3 were resistant.

General Information:

During the period covered by this report the following 5000 routine specimens were processed.

Water samples	1041
Urine specimens	231
Urethral specimens	247
Stool specimens	971
Dairy products	244
Throat swabs	197
Blood cultures	46
Sputum specimens	81
Pus & lesions	82
Cerebrospinal fluid	7
Miscellaneous cultures	155

Sera for

Heterophile Test	394
C-Reactive protein	29
Cold agglutinins	6
Febrile agglutinations	108
VDRL	1161

Table I

Enterobacteriaceae isolated from acute diarrhea cases in Thailand
from 1 April 1966 through 31 March 1967

Month	No. of Specimens	Salmonellae	Shigellae	Paracolons
April 1966	255	68	17	31
May	276	95	3	77
June	376	61	26	83
July	274	15	9	65
August	221	17	13	133
September	274	47	12	79
October	161	30	12	49
November	238	27	12	95
December	176	32	13	25
January 1967	210	21	10	56
February	222	33	16	58
March	252	22	12	30
Total	2935	468	155	781
Percentage of Total Specimens		15.9	0.53	26.6

Table II

Salmonella species isolated in Thailand from 1 April 1966 through 31 March 1967

Species	Group	Children	Adults	Unknown	Total
Salmonella derby	B	82	4	3	89
S. paratyphi B	B	256	10	8	274
S. saint paul	B	2	0	0	2
S. stanley	B	3	1	0	4
S. typhimurium	B	2	0	0	2
S. heidelberg	B	1	0	0	1
S. montevideo	C_1	18	0	1	19
S. tennessee	C_1	2	0	0	2
S. virchow	C_1	1	0	0	1
S. oslo	C_1	3	0	0	3
S. typhisuis	C_1	2	0	0	2
S. bovismorbificans	C_2	5	0	0	5
S. newport	C_2	8	1	0	9
S. typhosa	D	10	2	0	12
S. anatum	E_1	17	1	0	18
S. lexington	E_1	6	0	0	6
S. meleagridis	E_1	1	0	0	1
S. weltevreden	E_1	18	0	0	18
Total		437	19	12	468
Percentage of isolations		93.4	4.0	2.6	

Table III

Shigella species isolated in Thailand from 1 April 1966 through 31 March 1967

Species	Group	Children	Adults	Unknown	Total
Shigella dysenteriae 1	A	11	1	0	12
Sh. dysenteriae 2	A	1	0	0	1
Sh. flexneri 1	B	4	0	0	4
Sh. flexneri 2	B	27	6	2	35
Sh. flexneri 3	B	48	9	6	63
Sh. flexneri 4	B	8	0	0	8
Sh. flexneri 6	B	3	0	0	3
Sh. boydii 2	C	1	0	0	1
Sh. boydii 4	C	1	0	0	1
Sh. sonnei form I	D	23	1	2	26
Sh. sonnei form II	D	1	0	0	1
Total		128	17	10	155
Perceniage of isolations		82.6	11.0	6.4	

Table IV

Enteropathogenic Escherichia coli from Acute Diarrhea cases in Thailand from 1 April 1966 through 31 March 1967

	Thai nationals	Caucasians
Number examined	2062	169
Rough	1207	104
Negative	687	58
Positive	168	7
Serotypes		
025:B19:B23	52	2
026:B6	4	0
055:B5	1	0
086:B7	9	1
0112:B11	2	2
0119:B14	29	1
0125:B15	28	1
0126:B16	23	0
0127:B8	5	0
0128:B12	15	0

Table V

SENSITIVITY OF ENTERIC ORGANISMS TO TETRACYCLINE*
from 1 April 1966 through 31 March 1967

	No. of strains tested	Inhibited at mcg/ml									
		>200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B	21	6	14							1	
S. weltreveden	31	3	3			2	8	5	7	2	1
S. anatum	27				2	5	4	5	4	7	
S. oslo	4					1	1		1	1	
S. meleagridis	1						1				
S. derby	11							3	2	6	
S. bovismorbificans	3		1	1			1				
S. monte 'deo	4	3							1		
S. typhisuis	1						1				
S. stanley	6					1	3			2	
S. typhosa	14						6	4	2	2	
S. lexington	9	1					5	1		2	
S. manchester	2						2				
S. newport	10						5	2		3	
S. heidelberg	1	1									
S. virchow	2						1			1	
S. enteritidis	1							1			
S. sain paul	3	2							1		
S. thompson	1									1	
S. paratyphi C	2								2		
S. typhimurium	3	1						1	1		
S. tennessee	2						1				1
Sh. sonnei form I	29	2	3	5	8	5	5		1		
Sh. ,, ,, II	3	1			1				1		
Sh. flexneri 1	6	3	2			1					
Sh. ,, 2	33	1	2	7	11	1	6	5			
Sh. ,, 3	40		30	2	2	4	2	1			
Sh. ,, 4	13	4	2	1			1				
Sh. ,, 6	4			1	1	1				1	
Sh. dysenteriae 1	20		10	7	1		1		1		
Sh. ,, 2	1						1				
Sh. ,, 3	2							2			
Sh. boydii 2	1						1				
Sh. ,, 4	1						1				
Sh. ,, 5	1									1	
Sh. ,, 7	1									1	
Pathogenic E. coli 055:B5	2									2	
,, 026:B6	3	1	1			1					
,, 086:B7	3	3									
,, 0112:B11	7	3		1				2	1		
,, 0128:B12	9	4		1			2			2	
,, 0119:B14	6	4	1				1				
,, 0125:B15	21	14		2		1	1		1	2	
,, 0126:B16	12	5		2	3		1	1			
,, 0124:B17	4	2								2	
,, 025:B19:B23	25	15		1		1	4	2	1	1	

*Results are expressed as No. of isolates sensitive to each concentration

Table VI

SENSITIVITY OF ENTERIC ORGANISMS TO COLISTIN*
from 1 April 1966 through 31 March 1967

	No. of strain tested	Inhibited at mcg/ml									
		>200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B	21	1					2	4	2	7	5
S. weltreveden	31					—		2	17	10	2
S. anatum	27						1	6	9	10	1
S. oslo	4					1		3			
S. meleagridis	1									1	
S. derby	2							2	3	4	2
S. bovismorbificans	3						1	2			—
S. montevideo	4						1			3	
S. typhisuis	1							1			
S. stanley	6							1	4	1	
S. typhosa	14								1	3	10
S. lexington	9							2	3	2	2
S. manchester	2							2			—
S. newport	10						1	1	2	4	2
S. heidelberg	1					—				1	
S. virchow	2								1		1
S. enteritidis	1									1	
S. sain paul	3								2	1	
S. thompson	1									1	
S. paratyphi C	2							1			1
S. typhimurium	3							1	1	1	
S. tennessee	2							1		1	
Sh. sonnei form I	29				1			1	6	4	17
Sh. ,, ,, II	3										3
Sh. flexneri 1	6							1	1	1	4
Sh. ,, 2	32				3	1	3		1	4	20
Sh. ,, 3	39			1				2	1	8	27
Sh. ,, 4	13								1	4	8
Sh. ,, 6	5								1	2	2
Sh. dysenteriae 1	23				1			1	1	2	18
Sh. ,, 2	1									1	
Sh. ,, 3	2									2	
Sh. boydii 2	1							1			
Sh. ,, 4	1									1	
Sh. ,, 5	1										1
Sh. ,, 7	1										1
Pathogenic E. coli 055:B5	2						1		1		
,, 026:B6	3					1			1		1
,, 086:B7	3							1		1	
,, 0112:B11	7						1	2	2		2
,, 0128:B12	9			1				1	3	3	1
,, 0119:B14	6							1		2	3
,, 0125:B15	18		2		1	2		2	3	4	4
,, 0126:B16	12		2						2	4	4
,, 0124:B17	4								1	3	
,, 025:B19:B23	25		1				—	2	9	2	11

* Results are expressed as No. of isolates sensitive to each concentration

Table VII

SENSITIVITY OF ENTERIC ORGANISMS TO KANAMYCIN SULFATE*

from 1 April 1966 through 31 March 1967

	No. of strains tested	Inhibited at mcg/ml									
		> 200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B											
S. Weltreveden	22	4				1	14	2		1	
S. anatum	11					7	2	2			
S. oslo	2					1	1				
S. meleagridis	1						1				
S. derby											
S. bovismorbificans	3					1	2				
S. montevideo											
S. typhisuis	1						1				
S. stanley	4						4				
S. typhosa	8							7	1		
S. lexington	6					1	4	1			
S. manchester	2					2					
S. newport	2						2				
S. heidelberg	1	1									
S. virchow	1						1				
S. enteritidis											
S. sain paul	1	1									
S. thompson											
S. paratyphi											
S. typhimurium	1	1									
S. tennessee											
Sh. sonnei form I	12						10	2			
Sh. ,, ,, II	1						1				
Sh. flexneri 1	4					1		2			
Sh. ,, 2	24	2	2	1	3		4	12			
Sh. ,, 3	18		1					17			
Sh. ,, 4	5				1		1	3			
Sh. ,, 6	3		1				1	1			
Sh. dysenteriae 1	16				2		1	9	4		
Sh. ,, 2	1					1					
Sh. ,, 3	2							2			
Sh. boydii 2	1					1					
Sh. ,, 4	1							1			
Sh. ,, 5											
Sh. ,, 7											
Pathogenic E. Coli 055:B5											
,, 026:B6											
,, 086:B7	2	2									
,, 0112:B11	3	2				1					
,, 0128:B12	6	3				1	2				
,, 0119:B14	5	2				1	2				
,, 0125:B15	13	8				1	3	1			
,, 0126:B16	3	1					2				
,, 0124:B17	2	1					1				
,, 025:B19:B23	20	7				4	6	3			

*Results are expressed as No. of isolates sensitive to each concentration

Table VIII

SENSITIVITY OF ENTERIC ORGANISMS TO CHLORAMPHENICOL*

from 1 April 1966 through 31 March 1967

	No. of strains tested	Inhibited at mcg/ml									
		> 200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B	21	11	1						4	5	
S. Weltreveden	29		3	1			3	2	13	7	
S. anatum	27					1			17	9	
S. oslo	4								4		
S. meleagridis											
S. derby	11							2	1	8	
S. bovismorbificans	3		2						1		
S. montevideo	4	4									
S. typhisuis	1								1		
S. stanley	5								3	2	
S. typhosa	12								2	10	
S. lexington	9							1	3	5	
S. manchester	2							2			
S. newport	9							2	4	3	
S. heidelberg	1								1		
S. virchow	2							1	1		
S. enteritidis	1									1	
S. sain paul	3		1					1	1		
S. thompson	1								1		
S. paratyphi C	2									2	
S. typhimurium	3								1	2	
S. tennessee	2									2	
Sh. sonnei form I	29	8	13	1	1		2	2	1	1	
Sh. ,, ,, II	3	1	2								
Sh. flexneri 1	6	1	2	1	2						
Sh. ,, 2	32	1	2	10	11	1			5	3	
Sh. ,, 3	29	1	2	16	15	4				1	
Sh. ,, 4	13	1		7	4	1					
Sh. ,, 6	5			1			1			3	
Sh. dysenteriae 1	23			3	16	1	1	1		1	
Sh. ,, 2	1							1			
Sh. ,, 3	2						2				
Sh. boydii 2	1								1		
Sh. ,, 4	1						1				
Sh. ,, 5	1									1	
Sh. ,, 7	1									1	
Pathogenic E. coli 055:B5	2									2	
,, 026:B6	3			1	1					1	
,, [illegible]B7	4		1		2				1		
,, [illegible]B11	7	1		1				2		3	
,, [illegible]B12	9	2	3	2			1			1	
,, [illegible]9:B14	5		4	1							
,, 0125:B15	21	4	4	1		2			4	6	
,, 0126:B16	12	4	3		2	1	1	1			
,, 0124:B17	4		2							2	
,, 025:B19:B23	24	5	9	2			3	3	1	1	
					1						

* Results are expressed as No. of isolates sensitive to each concentration

Table IX

SENSITIVITY OF ENTERIC ORGANISMS TO NEOMYCIN SULFATE*

from 1 April 1966 through 31 March 1967

	No. of strains tested	Inhibited at mcg/ml									
		>200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B	21	20	—	—	—	—	1	—	—	—	—
S. weltreveden	31	5	—	—	—	—	12	11	3	—	—
S. anatum	27	—	—	—	2	1	15	6	1	—	—
S. oslo	4	—	—	—	—	2	—	1	1	—	—
S. meleagridis	1	—	—	—	—	—	1	—	—	—	—
S. derby	11	—	—	—	—	—	4	1	6	—	—
S. bovismorbificans	3	—	—	—	—	—	2	1	—	—	—
S. montevideo	4	3	1	—	—	—	—	—	—	—	—
S. typhisuis	1	—	—	—	—	—	1	—	—	—	—
S. stanley	6	—	—	—	—	—	2	4	—	—	—
S. typhosa	12	1	—	—	—	—	—	4	4	3	—
S. lexington	9	—	—	—	—	—	4	3	1	1	—
S. manchester	2	—	—	—	—	—	—	1	1	—	—
S. newport	9	—	—	2	—	—	3	4	—	—	—
S. heidelberg	1	1	—	—	—	—	—	—	—	—	—
S. virchow	2	—	—	—	—	—	2	—	—	—	—
S. enteritidis	1	—	—	—	—	—	—	—	1	—	—
S. sain paul	3	2	—	—	—	—	1	—	—	—	—
S. thompson	1	—	—	—	—	—	—	1	—	—	—
S. paratyphi C	2	—	—	—	—	—	1	—	—	1	—
S. typhimurium	3	1	—	—	—	—	—	2	—	—	—
S. tennessee	2	—	—	—	—	—	—	1	—	1	—
Sh. sonnei form I	28	—	—	—	—	—	2	19	2	5	—
Sh. ,, ,, II	3	—	—	—	—	—	2	—	1	—	—
Sh. flexneri 1	6	—	—	—	—	—	—	4	2	—	—
Sh. ,, 2	33	—	—	—	1	—	—	22	10	—	—
Sh. ,, 3	40	—	—	—	—	—	—	13	20	7	—
Sh. ,, 4	13	—	—	—	—	—	—	6	6	1	—
Sh. ,, 6	5	—	—	—	—	—	1	2	2	—	—
Sh. dysenteriae 1	23	—	—	—	—	1	—	1	16	5	—
Sh. ,, 2	1	—	—	—	—	—	1	—	—	—	—
Sh. ,, 3	2	—	—	—	—	—	—	2	—	—	—
Sh. boydii 2	1	—	—	—	—	—	1	—	—	—	—
Sh. ,, 4	1	—	—	—	—	—	—	—	1	—	—
Sh. ,, 5	1	—	—	—	—	—	—	1	—	—	—
Sh. ,, 7	1	—	—	—	—	—	—	—	1	—	—
Pathogenic E. coli 055:BS	2	1	—	—	—	—	1	—	—	—	—
,, 026:B6	3	1	—	—	—	—	1	1	—	—	—
,, 086:B7	3	2	—	—	—	—	—	1	—	—	—
,, 0112:B11	6	—	—	1	—	1	3	1	—	—	—
,, 0128:B12	10	3	1	—	—	—	6	—	—	—	—
,, 0119.B14	6	3	—	—	—	—	3	—	—	—	—
,, 0125:B15	21	10	—	1	—	—	6	3	—	1	—
,, 0126:B16	12	10	—	—	—	—	2	—	—	—	—
,, 0124:B17	4	1	—	—	—	—	3	—	—	—	—
,, 025:B19:B23	25	8	—	—	—	—	7	8	2	—	—

* Results are expressed as No. of isolates sensitive to each concentration

Table X

SENSITIVITY OF ENTERIC ORGANISMS TO NALIDIXIC ACID*
from 1 April 1966 through 31 March 1967

	No. of strain tested	Inhibited at mcg/ml									
		> 200	200	100	50	25	12.5	6.25	3.12	1.56	0.78
S. paratyphi B	21	—	—	—	—	—	—	2	5	3	11
S. anatum	16	—	—	—	—	—	—	3	4	2	7
S. newport	7	—	—	—	—	—	—	—	—	3	4
S. weltreveden	9	—	—	—	—	—	—	1	1	3	4
S. derby	11	—	—	—	—	—	—	—	2	1	8
S. stanley	2	—	—	—	—	—	—	—	—	—	2
S. montevideo	4	—	—	—	—	—	—	2	2	—	—
. lexington	3	—	—	—	—	—	—	—	—	3	—
Sh. sonnei form I	17	—	—	—	—	—	—	—	1	6	10
Sh. ,, ,, II	2	—	—	—	—	—	—	—	1	1	—
Sh. flexneri 1	2	—	—	—	—	—	—	—	—	—	2
Sh. ,, 2	9	—	—	—	—	—	—	—	2	3	4
Sh. ,, 3	22	—	—	—	—	—	—	—	6	3	13
Sh. ,, 4	8	—	—	—	—	—	—	—	1	2	5
Sh. ,, 6	2	—	—	—	—	—	—	—	—	—	2
Sh. boydii 5	1	—	—	—	—	—	—	—	—	1	—
Sh. ,, 7	1	—	—	—	—	—	1	—	—	—	—
Sh. dysenteriae 1	9	—	—	—	—	—	—	—	—	2	7
Pathogenic E. coli 055:B5	2	—	—	—	—	—	1	1	—	—	—
,, 026:B6	3	—	—	—	—	—	—	2	—	—	1
,, 086:B7	1	—	—	—	—	—	—	—	—	1	—
,, 0112:B11	5	—	—	—	—	—	—	—	2	1	2
,, 028:B12	4	—	—	—	—	—	—	—	—	—	4
,, 0119:B14	1	—	—	—	—	—	—	—	—	—	1
,, 0126:B16	9	—	—	—	—	—	—	—	—	4	5
,, 0124:B17	2	—	—	—	—	—	—	1	1	—	—
,, 025:B19:B23	4	—	—	—	—	—	—	1	1	—	2

*Results are expressed as No. of isolates sensitive to each concentration

Table XI

Survey of Enteric Pathogens at Central Preventorium for Children
Nondhburi Province, Thailand July 1966

Total subject	227 — Adults 75	
	— Children 152*	
Total stool specimens	247	
Specimens from Fomites	21	

Source of Specimens	Total specimens	Pathogenic E. coli	Salmonella	Shigella
Children without Diarrhea	136	22 (16.2%)	10 (7.4%)	1 (.07%)
Children with Diarrhea	36	9 (25%)	6 (16.7%)	0
Adults without Diarrhea	73	8 (11.0%)	10 (13.7%)	3 (4.1%)
Adults with Diarrhea	2	0	1 (50%)	0
Fomites	21	2	0	0

* 20 subjects had 2 cultures examined.

Table XII
Survey of Enteric Pathogens at Central Preventorium for Children
Nondhburi Province, Thailand, July 1966

Source of specimens	Ward	Total Specimens	Pathogenic E. coli		Salmonella		Shigella	
			Group	No.	species	No.	species	No.
Children without Diarrhea	A	51	C 0125:B15 C 0128:B12 C 025:B19:B23	2 1 6	anatum lexington derby montevideo	1 2 1 1	flexneri 2	1
	B	85	C 0125:B15 C 025:B19:B23	1 12	anatum lexington montevideo	1 1 3		
Children with	A	10	C 025:B19:B23	1	lexington newport weltevreden	1 1 1		
	B	26	C 0125:B15 C 025:B19:B23	3 5	weltevreden montevideo	1 2		
Adults without Diarrhea	A	34	B 0124:B17 C 0125:B12 C 025:B19:B23	2 2 2	anatum lexington derby typhimurium	2 1 2 1	flexneri 2 dysenteriae 1	1 1
	B	39	B 086:B7 C 025:B19:B23	1 1	typhimurium derby lexington weltevreden	1 1 1 1	boydii	1
Adults with Diarrhea	A B	0 2			oslo	1		
Fomites	A B	10 11	C 025:B19:B23 C 025:B19:B23	1 1				

Table XIII

Survey of Enteric Bacterial Pathogens in Nakornpanom, Thailand
(May 1966)

Source of specimens	Total	Path. *E. Coli*	Shigellae	Salmonellae
Thai adults without diarrhea	56	2	0	0
Thai adults with diarrhea	24	0	3	2
Thai children without diarrhea	38	1	0	2
Thai Children with diarrhea	11	1	4	1

Table XIV

Survey of Intestinal Parasites in Nakornpanom, Thailand (16 May - 19 May 1966)

Sources of Specimens	No. of Specimens examined	No. of Positive Specimens	Helminths					Protozoa			No parasites seen
			Nematoda				Trematoda	Intestinal			
			Ascaris Lembricoides	Trichuris Trichina	Hookworm	Strongeloides stercoralis	Opistorchis	Entamoeba coli	Entamoeba histolyiica	Chilomastri-semesnili	
Normal Thai adults A	45	24	1	2	3	2	13	5	2	–	21
Thai adults with diarrhea G	9	4	–	–	–	–	–	3	1	–	5
Normal Thai children F	33	12	1	1	2	0	5	4	–	1	21
Thai children with diarrhea E	9	2	–	–	–	–	–	2	–	–	7

Table XV

Enteropathogens recovered from Praputhabath hospital patients using Sander's methodology

Total Specimens	Adults	Children	Salmonellae	Shigellae	Enteropathogenic E. Coli
141	34	107	13	13	14

Distribution of species

Salmonellae

S. typhimurium	7
S. saint paul	6

Shigellae

Sh. dysenteriae 1	1
Sh. flexneri 1	2
Sh. flexneri 3	7
Sh. flexneri 4	1
Sh. sonnei form II	1
Alkalescens-dispar 04	1

E. E. coli

025:B19:B23	2
026:B6	1
055:B5	1
086:B7	1
0119:B14	2
0124:B17	1
0125:B15	2
0128:B12	4

Table XVI

Culture Media Used for Enumeration of Intestinal Aspirates

Medium	Specific for	Incubation condition (37°C)	
		Time (Hrs)	Environment
Blood Agar	Total aerobes	24	aerobib
MacConkey Agar	Total gram negative aerobes	24	aerobic
Mannitol Salt Agar	Staphylococci	24	aerobic
SF Agar	Fecal Streptocci	96	aerobic
Tellurite*	vibrio	24	aerobic
Sabouraud Dextrose Agar	Yeast	24	aerobic
Blood Agar	Total Anaerobes	48	anaerobic
Neomycin Blood Agar	Total gram neg. anaerobes	48	anaerobic
Lactobacillus	Lactobacilli	48	anaerobic

*Alkaline lauryl sulfate tellurite

Table XVII

Bacterial Growth from Upper Intestinal Aspirates in Normal Thai Adults
during Lactose Tolerance Study

Bacteria	Percentage Recovery Pre-lactose		Percentage Recovery Post-lactose	
	10^2-10^7	$> 10^3$	10^2-10^7	$> 10^3$
Coliform	11.63	4.65	14.58	8.33
Staphylococci	32.56	4.65	22.92	0
Streptococci	44.19	32.56	31.25	25.00
Enterococci	23.26	2.33	18.75	4.17
Diphtheroids, Bacillus	34.88	2.33	35.42	8.33
Proteus, Pseudomonas	6.98	0	2.08	2.09
Veillonella	4.65	0	10.41	2.09
Neisseria	18.61	18.60	18.75	8.33
Clostridia	0	0	0	0
Bacteroides	0	0	0	0
Lactobacilli	2.33	0	2.08	0
Yeast	30.23	2.33	33.33	2.09

Table XVIII

Comparison of Quantitative Bacterial Flora of Intestinal Aspirates

Bacteria	Period of study	Total specimens	Range	Median
Coliform	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^4 $<10^2$ to 10^5	$<10^2$ $<10^2$
Proteus sp., Pseudomonas sp.,	Pre-lactose Post-lactose	43 48	$<10^2$ to $10^{[illegible]}$ $<10^2$ to 10^4	$<10^2$ $<10^2$
Lactobacilli	Pre-lactose Post-lactose	43 48	$<10^2$ to $10^{[illegible]}$ $<10^2$ to $10^{[illegible]}$	$<10^2$ $<10^2$
Diphtheroids, Bacillus sp.	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^4 $<10^2$ to 10^4	$<10^2$ $<10^2$
Veillonella sp.	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^3 $<10^2$ to 10^4	$<10^2$ $<10^2$
Neisseria sp.	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^6 $<10^2$ to 10^6	$<10^2$ $<10^2$
Streptococci	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^6 $<10^2$ to 10^7	$<10^2$ $<10^2$
Enterococci	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^4 $<10^2$ to 10^5	$<10^2$ $<10^2$
Staphylococci	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^5 $<10^2$ to $10^{[illegible]}$	$<10^2$ $<10^2$
Yeast	Pre-lactose Post-lactose	43 48	$<10^2$ to 10^4 $<10^2$ to 10^4	$<10^2$ $<10^2$

Table XIX

Comparison of Frequency Distribution of Bacterial Counts of Intestinal Aspirates

Bacteria		Bacterial counts/ml of Intestinal Fluid						
		$<10^2$	10^2	10^3	10^4	10^5	10^6	10^7
Coliform	Pre Post	38 41	1 0	2 3	2 3	0 1	0 0	0 0
Proteus, Pseudomonas	Pre Post	40 47	3 0	0 0	0 1	0 0	0 0	0 0
Lactobacilli	Pre Post	42 47	0 1	1 0	0 0	0 0	0 0	0 0
Diphtheroids, Bacillus	Pre Post	28 31	12 10	2 3	1 3	0 1	0 0	0 0
Veillonella	Pre Post	41 43	1 2	1 2	0 1	0 0	0 0	0 0
Neisseria	Pre Post	35 39	0 1	0 5	3 1	3 2	2 0	0 0
Streptococci	Pre Post	24 33	1 0	4 3	4 7	5 1	5 3	0 1
Enterococci	Pre Post	32 39	4 5	6 2	1 1	0 1	0 0	0 0
Staphylococci	Pre Post	29 37	8 11	4 0	1 0	1 0	0 0	0 0
Yeast	Pre Post	30 32	7 14	5 1	1 1	0 0	0 0	0 0

Table XX

The effects of Neomycin* on bacterial flora of normal upper G-I tract

No. of subjects	No. of specimens		# specimens with significant growth		Organisms detected	
	Pre Rx	Post Rx	Pre Rx	Post Rx	Pre Rx	Post Rx
7	17	11	2***	0	Streptococcus	Streptococcus
					Staphylococcus	Staphylococcus
					Diphtheroids	Diphtheroids
					Yeast	Yeast
					Bacillus sp.	Proteus sp.
					Lactobacilli	Coliform
					Pseudomonas	Pseudomonas

* Total of 4 grams in 24 hours

** More than 10[illegible] count/ml

*** Significant growth of streptococci

Table XXI

Summary of Antibiotic producing E. coli isolated from children under 2 years of age*

Group	No. of individuals	No. of specimens	No. of E. coli strains	Distribution of E. coli strains**						antagonists/ E. coli tested		Specimens c antagonists/ Total specimens	
				0	(%)	+	(%)	++	(%)				
A Normal subjects	9	28	260	241	92.70	18	6.92	1	0.38	19/260	7.30%	6/28	21.43%
B Clinically ill, positive salmonella	3	13	122	121	99.28	0	0	1	0.82	1/122	0.82%	1/13	7.69%
C Clinically ill, salmonella and Path. E. coli	5	17	154	149	96.73	1	0.65	4	2.54	5/154	3.24%	4/17	23.53%
D Clinically ill, shigella positive	7	30	234	189	80.77	36	15.39	9	3.84	45/234	19.23%	10/30	33.33%
E Clinically ill, shigella and salmonella	5	23	192	142	73.59	3	1.56	47	24.85	50/192	26.41%	9/23	39.12%

0 Inactive
\+ Slightly active
++ Moderately active

* Specimens from Salmonellae carriers and diarrhea of unknown etiology are not included.
** as tested with standard Sh. sonnei form I indicator strain.

Table XXII

Follow up cultures from clinically ill patients from whom both Shigellae and Salmonellae were isolated

Subject (organisms isolated)	Date of onset	Culture Date	# E. coli strains* 0	+	++	Total E.coli strains 0	+	++	Total antagonists / Total No. E. coli
PD 11	15 Feb 67	21 Feb 67	0	0	10				
		22 Feb 67	0	0	10	9	0	31	77.50%
(Sh. flexneri 3,		23 Feb 67	0	0	10				
S. paratyphi B)		1 Mar 67	9	0	1				
PD 12	Unknown	21 Feb 67	10	0	0				
(Sh. flexneri 3,		22 Feb 67	2	0	5	22	0	5	18.52%
S. paratyphi B)		23 Feb 67	10	0	0				
PD 4	21 Jan 67	25 Jan 67	10	0	0				
		26 Jan 67	10	0	0	42	0	8	16.00%
(Sh. flexneri 1,		27 Jan 67	11	0	0				
S. paratyphi B)		30 Jan 67	1	0	4				
		3 Feb 67	4	0	0				
		7 Feb 67	6	0	4				
PD 14	25 Feb 67	28 Feb 67	6	0	2				
		1 Mar 67	9	0	1	44	0	3	6.38%
(Sh. boydii 4,		2 Mar 67	12	0	0				
S. paratyphi B,		6 Mar 67	10	0	0				
E. E. coli-B:086:B7)		8 Mar 67	3	0	0				
		17 Mar 67	4	0	0				
PD 19	1 Mar 67	7 Mar 67	10	0	0				
		8 Mar 67	2	3	0	25	3	0	10.71%
(Sh. flexneri 3,		9 Mar 67	3	0	0				
S. paratyphi B)		14 Mar 67	10	0	0				

Ave. 20.7%

0 = Inactive
\+ = Slightly active
++ = Moderately

* as tested with standard Sh. sonnei form I indicator strain.

Table XXIII

Follow up cultures of clinically ill patients from whom Shigellae were isolated

Subject (organisms isolated)	Date of onset	Culture Date	# E. coli strains* 0	.	. .	Total E. coli strains 0	.	. .	Total antagonists / Total No. E. coli
PN 6 (Sh. sonnei form I)	27 Feb 67	27 Feb 67	3	0	0	10	3	0	23.08%
		28 Feb 67	2	0	0				
		2 Mar 67	5	3	0				
PN 5 (Sh. sonnei form I)	2 Mar 67	8 Mar 67	6	0	0	26	0	0	0%
		9 Mar 67	10	0	0				
		13 Mar 67	10	0	0				
PD 6 (Sh. boydii 6)	1 Jan 67	27 Jan 67	0	10	0	31	12	1	29.54%
		1 Feb 67	9	0	0				
		7 Feb 67	5	0	0				
		13 Feb 67	9	0	1				
		21 Feb 67	8	2	0				
PD 7 (Sh. flexneri 3)	26 Jan 67	3 Feb 67	10	0	0	34	1	0	2.85%
		6 Feb 67	10	0	0				
		8 Feb 67	2	1	0				
		9 Feb 67	2	0	0				
		17 Feb 67	10	0	0				
PD 8 (Sh. flexneri 3)	5 Feb 67	6 Feb 67	10	0	0	22	0	0	0%
		8 Feb 67	10	0	0				
		9 Feb 67	2	0	0				
PD 9 (Sh. dysenteriae 1)	Unknown	9 Feb 67	4	0	0	23	20	1	47.72%
		10 Feb 67	10	0	0				
		14 Feb 67	9	0	1				
		16 Feb 67	0	10	0				
		21 Feb 67	0	10	0				
PD 10 (Sh. sonnei form I)	18 Feb 67	22 Feb 67	10	0	0	42	0	7	14.29%
		23 Feb 67	4	0	0				
		28 Feb 67	6	0	0				
		2 Mar 67	7	0	3				
		6 Mar 67	8	0	0				
		8 Mar 67	7	0	4				

Ave. 19.3%

0 Inactive

. Slightly active

. . Moderately

* as tested with standard Sh. sonnei form I indicator strain.

Table XXIV

Comparison of Incidence of Antagonistic *E. coli* among non-diarrhea subject. in North America and Bangkok

Group	Age	Total strains E. coli	Coliform strains							
			0	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	& [illegible]	[illegible]
N. Y. State[1]	5-22 yrs	2648	2339	88.30	192	7.30	117	4.40	309	11.70
North Carolina State[2]	Adults	2105	1583	75.20	408	19.40	114	5.40	522	24.80
South Texas[3]	Under 10 yrs	1243	1015	81.70	104	8.3	124	10.00	228	18.30
Bangkok	Under 2 yrs	260	241	92.8	18	6.74	1	0.46	19	7.20

1 Halbert, S. P. 1948. The relation of antagonistic coliform organisms to shigella infections. J. Immunol. 60:(1), 1948.

2. Halbert, S. P., and M. Gravatt. Prevalence of antibiotic - producing coliform organisms. Public Health Reports, 64:(10), 313-318, 1949.

3. Halbert, S. P. The antagonism of coliform bacteria against shigellae. J. Immunol. 58: 1948.

Table XXV

Comparison of Incidence of Antagonistic E. Coli among Patients with shigellosis in New York State and Bangkok

Group	Age	Total strains coliform	Coliform strains							
			0	%	+	%	++	%	+ & ++	%
N.Y. State[1]	5-22 yrs	1578	1012	64.10	383	24.27	183	11.63	566	35.90
Bangkok	Under 2 yrs	234	189	80.70	36	15.39	9	3.84	45	19.23

1 Halbert, S.P. The relation of antagonistic coliform organisms to shigella infections. J. Immunol. 60: (3), 1948.

Title:	Relation of Ceroid to Small Bowel Function
Principal Investigator:	Captain Frank J. Troncale, MC
Associate Investigators:	Captain Louis H. Miller, MC Lt. CDR Gerald T. Keusch, USPHS Dhira Sonakul Comer, M.D. Kasem Chittayasothorn, M.D.
Period of Report:	1 November 1965 31 March 1967

OBJECTIVE: Deposition of ceroid, a lipofucsin pigment, was recently found in 76% of 139 unselected autopsies from Udorn Provincial Hospital in Northeast Thailand[1]. The pigment was deposited predominantly in the smooth muscle of the gastrointestinal tract and to a lesser extent in smooth muscle of blood vessels, prostate, uterus, urinary bladder, and in the lymph nodes. A similar distribution of ceroid pigment has been described in malabsorptive states such as cystic fibrosis of the pancreas[2], chronic pancreatitis[3], and non-tropical sprue[4-6]. Because of this, the present study was undertaken to search for malabsorptive disease in North-east Thailand.

DESCRIPTION:

Methods:

Clinical Material: 63 patients were studied on the wards of the Udorn Provincial Hospital. Selection was based on willingness to cooperate. Patients with severe anemia or recent surgery were rejected from the study. There were 38 males and 27 females ranging in age from 14 to 59 years (mean age 32). Because of limited laboratory and x-ray facilities, only a tentative diagnosis based on history and physical examination performed at the time of study was possible (see Table I). The following studies were performed on most of the patients: upper gastrointestinal biopsies, d-xylose tolerance test, vitamin A tolerance test, and serum vitamin E, albumin, cholesterol, and B-carotene. In addition, 3-day fecal fat collections were carried out in 8 patients. Biopsies were taken with a Crosby-kugler capsule. After swallowing the capsule, the patients were turned on their right sides for 20-30 minutes, and then the capsule was fired. There were no complications from this procedure. Formalin-fixed tissue was stained with hematoxylin and eosin, PAS, Ziehl-Neelsen and brilliant green.

The 5 hour d-xylose urinary excretion was measured in 43 patients after a 25 g oral dose, and in 15 after a 5 g dose. The 5 hour urine volume was greater than 170 ml in all. Two-hour serum xylose levels were also performed in those given the 25 g dose.

Serum vitamin A levels were measured fasting and 5 hours after an oral dose of 250,000 IU diluted to 5 ml with vegetable oil.

Three-day fecal fat collections were marked with carmine red. The diet was supplemented with 75 g of butter daily.

Serum, urine, and stool specimens were collected by metabolic nurses, and were frozen in dry ice. Methods were the same as those employed in a previous study of normal adult Thais living near Bangkok[7].

Results

Gastrointestinal Biopsies:

63 biopsies were obtained from the upper gastrointestinal tract (see Table II). 19 out of 35 antral biopsies and 4 out of 4 esophageal biopsies contained ceroid pigment, whereas none of the 19 from the fundus and body of the stomach, duodenum or jejunum were positive. Five other biopsies were considered unsatisfactory for determining the presence or absence of ceroid because smooth muscle (muscularis mucosae) was not present. The distribution of the pigment in the upper gastrointestinal tract thus confirms that found in the autopsy series from this hospital( ) where ceroid occurred in the esophagus in 75% of the cases examined, and further, was found in the esophagus in every case where ceroid was also found elsewhere in the body. In contrast, the gastric antrum contained the pigment in only 46% of the cases, and only 58% of the time when some other site or sites were also affected. It is thus likely that many of the patients in the present study whose gastric antrum biopsies were ceroid-negative were actually ceroid-positive. Therefore, it is more meaningful when making comparisons between ceroid-positive and ceroid-negative patients to consider the entire Udorn stud· patients as a homogeneous ceroid-positive group and the previously-studied Bangkok subjects as a ceroid-negative group. However, because there is such a paucity of information on this subject, comparisons between ceroid-positive and ceroid-negative within the Udorn patient population, admittedly less valid, are also made. Because the incidence of ceroid in fundus and body of the stomach, and in the duodenum is so low (13% and 6%), it is unlikely that any positive cases were missed, and hence they are not included in comparison between ceroid-positive and ceroid-negative patients. Histologic grading of the amount of pigment, as previously described( ) is shown in Table III. No correlation was found between the amount of pigment deposited and the tests of absorption that follow.

D xylose Excretion Test:

The average 5 hour urinary xylose excretion after a 25 g dose in forty-four patients was 5.1 · 1.7* g. This group comprises all the subjects studied including those whose biopsies were unsatisfactory for evaluating the presence or absence of ceroid. This value was not significantly different from normal Bangkok subjects (see Table IV). The 2 hours serum xylose was also similar in the two groups.

In those from Udorn with the 5 g d-xylose dose, the 5 hour excretion was 1.49 · 0.39 g. Although we have no data from normal Thais in Bangkok using this dose for comparison, none of the Udorn patients excreted less than 1 g, the level observed in sprue patients( ).

After the 25 g dose, d-xylose excretion was 5.1 g in thirteen Udorn Hospital subjects with ceroid-positive biopsies, in comparison to 6.1 g in 10 subjects with ceroid-negative-negative biopsies. The difference is not significant (see Table V). Likewise, there was no difference in d-xylose excretion following the 5 g dose between the ceroid-positive and negative-patients. The two-hour serum-xylose value was, however, significantly higher (p .05) in the ceroid-negative group (50.9 mg%) than in the ceroid-positive subjects (41. 8 mg%).

Fecal fat:

Fecal fat excretion in 8 patients averaged 3.7 g per day. This value was not different from fat excretion in the Bangkok subjects (2.4 1.4 g). One of the Udorn patients had mildly elevated fat excretion (7.5 g/day). However, in this patient the xylose excretion (8.1 g), vitamin A tolerance, B-carotene, and hemotocrit were all normal.

* All values in the results are given as the mean · 1 S.D.

Vitamin A absorption:

Vitamin A malabsorption* occurred in 14 of 54 Udorn subjects tested (26%), compared to the 3 out of 27 in the Bangkok subjects (11%). This is not significantly different (see Table IV). There was also no difference in vitamin A absorption between the ceroid-positive and-negative patients in Udorn.

Biochemical tests:

Serum B-carotene values in 63 Udorn patients averaged 66 ± 35 ug% and were lower than in the Bangkok subjects (133 ± 65 ug%) (Table IV). There was no difference between ceroid-positive and ceroid-negative patients (Table V).

Serum albumin was also lower in the Udorn patients, when compared to the Bangkok subjects (Table IV). The difference between ceroid-negative and ceroid-positive patients, however, was insignificant (Table V).

The serum cholesterol values in the Udorn patients were the same as the Bangkok subjects (Table IV). As with the Bangkok group, no correlation was found between serum cholesterol and urinary d-xylose[11].

Discussion:

The present study confirms the previously reported distribution and incidence of ceroid pigment in the upper gastrointestinal tract of patients coming to autopsy at the Udorn provincial Hospital in northeast Thailand[1]. Because malabsorptive diseases have been found so frequently in the past in association with deposition of ceroid pigment, one would expect to find evidence of it in this population where 75% of the people have the pigment. Standard tests of intestinal absorption, however, failed to uncover any such examples, neither when the entire patient population studied was compared to Bangkok controls, where ceroid is rare, nor when the patient population with ceroid-positive and ceroid-negative biopsies were compared.

The mechanism of ceroid formation in vivo is not entirely clear. In experimental animals, pigment deposition can be induced by feeding vitamin E deficient diets[11,12]. The process can be hastened if, in addition, a diet high in usaturated fat is used. Ceroid is thus though to be formed when an antioxidant-deficient state occurs, but the exact mechanism is not known.

In clinical malabsorptive states accompanied by steatorrhea, malabsorption of vitamin E itself is likely to be the main factor contributing to pigment deposition. In the group of patients that we have studied, there is little evidence that malabsorption is present; thus, either a low dietary intake of vitamin E is present or an increased amount of unsaturated fat is responsible. While both factors are probably operative, the evidence for the latter is stronger based on present knowledge. It is known, for instance, that the unsaturated fat intake in the population in northeast Thailand is quite high, since the principle dietary staples are fermented fish and glutinous rice, the fish containing most of its fat in the unsaturated form[13]. Further evidence that increased unsaturated fat in the diet is playing a role is supported by the significantly lower serum total cholesterol in the ceroid-positive patients, since this type of dietary fat is known to be an effective cholesterol-lowering agent.

* Vitamin A malabsorption is defined as a rise of serum vitamin A at 5 hours of less than 125 ug% over the fasting value[9].

Table I Clinical Diagnoses of Subjects Studied at Udorn Provincial Hospital.

Accidental Trauma Gastrointestinal	12
Dysentery, convalescent	4
RUQ Pain, undiagnosed	2
Intestinal flukes	1
Appendiceal abscess	1
Musculoskeletal	7
Post-partum	6
Genitourinary	7
Endecrine (Thyrotoxicosis, Post-menopausal)	3
FUO, convalescent	3
Inguinal hernia	4
Anemia, mild, uncharacterized	2
Psychoneurosis	2
Malnutrition	2
Miscellaneous	9
Total	65

Table II Biopsy site and presence or absence of ceroid pigment in specimens from patients at Udorn Provincial Hospital, Thailand.

	Total Number	Ceroid-Positive	Ceroid-Negative
Stomach*			
Antrum	36	19	16
Fundus and Body	18	0	14
Esophagus	4	4	0
Duodenum and Jejunum	5	0	5
Total	63	23	35

Table III. Histologic grading of ceroid-positive biopsies.

		Number of positive biopsies Antrum	Esophagus
1+	Trace to small amount.	10	1
2	3-6 smooth muscle fibers affected in most high-power fields.	1	1
3	Many fibers pigmented; some fibers bulging with pigment.	6	1
4	Almost all smooth-muscle fibers heavily laden with pigment.	2	1
	Total	19	4

* 1 antral and 4 fundal biopsies had no muscularis mucosal, and therefore could not be evaluated for the presence of ceroid pigment.

Table IV. Comparison of Absorption Tests and Other Biochemical Data between Patients at the Udorn Provincial Hospital and Normal Thais in Bangkok, Thailand.

Location	25 g. d-xylose		Fecal Fat*	Vitamin A absorption, % abnormal +	Serum B carotene, ug/100 ml	Total serum cholesterol, mg/100 ml	Serum albumin, g/100 ml
	Urinary excretion g/5 hrs	2 hr serum xylose mg/100 ml					
Udorn	5.1 ± 1.7(44)++	43.4 ± 11.8(43)	3.7 ± 2.2 (8)	26 (54)	66 ± 35 (59)	171 ± 49 (59)	3.64 ± 0.56 (43)
Bangkok	5.8 ± 1.5(36)	39.8 ± 7.5(31)	2.3 ± 1.4 (35)	11 (27)	133 ± 65 (34)	177.5 ± 31.0(35)	4.2 ± 0.57 (32)
p value	N.S.	N.S.	N.S.	N.S.	<0.01	N.S.	< 0.01

* The diet was supplemented with 75 g of butter per day.

\+ The vitamin A absorption was considered abnormal if the increase over the fasting value was less than 125 μg/100 ml.

++ Mean ± S.D. The numbers in parenthesis are the number of subjects studied.

Table V. Comparison of absorption tests and other biochemical data between ceroid-positive and ceroid-negative patients at the Udorn Provincial Hospital, Thailand.

	Ceroid Positive	Ceroid Negative	P
No. of individuals*	23	16	
Males	14	5	
Females	9	11	
Average age years	33.7	28.8	
d-xylose excretion, g/5 hrs**	5.1 ± 1.61 (13)†	6.1 ± 1.55 (10)	N.S.
2 hr. serum xylose mg/100 ml	41.8 ± 12.2 (14)	50.9 ± 9.4 (10)	0.05
d-xylose excretion, g/5 hrs ††	1.45 ± 0.29 (6)	1.58 ± 0.53 (4)	N.S.
Vitamin A absorption, % abnormal§	26 (23)	12.5 (16)	N.S.
Fecal fat g/day≠	3.3 (3)	4.0 (3)	N.S.
Serum B-carotene, μg/100 ml	59 ± 43.5 (23)	78 ± 31.2 (16)	N.S.
Serum total cholesterol, mg/100ml	152 ± 37.3 (21)	205 ± 44.1 (15)	<0.01
Serum albumin, g/100 ml	3.55 ± 0.75 (14)	3.72 ± 0.59 (11)	N.S.

* Only those patients whose biopsies were from antrum or esophagus and contained muscularis mucosa are included here.

** 25 g. oral dose.

† MEAN ± S.D. The numbers in parentheses are the number of subjects studied.

†† 5 g. oral dose.

§ The vitamin A absorption was considered abnormal if the increase over the fasting value was less than 125/μg/100 ml.

≠ The diet was supplemented with 75 g. of butter per day.

Title: Study of Intestinal Immunoglobulins.

Principal Investigator: Captain Andrew G. Plaut, MC

Associate Investigators: Major Pipat Juttijudata, MC, RTA
Anong Intavivat, M.Sc. (Microbiology)
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Captain Frank J. Troncale, MC

Advisors: Richard A. Finkelstein, Ph.D.
Lt. Colonel Philip K, Russell, MC
Lt. Colonel Dale R. Snyder, MC
Bernhardt W. Langer, Ph.D.

Objective: Recent experimental work in other laboratories has shown that external body secretions (e.g. tears, nasal secretions, saliva and intestinal juice) contain immunoglobulins, principally of the IgA type. The role of these globulins is not known at the present time, although antibody activity has been demonstrated in nasal secretions following viral illness in man, and in stool filtrates of cholera patients. The purpose of the work described here is 1) to try to establish the type, and amount, of immunoglobulins in the intestinal juice of Thai subjects and 2) to determine if the normal patterns change in patients during episodes of intestinal diarrhea.

Description: Experiments performed to date have been directed at overcoming technical difficulties encountered in the study of intestinal juice immunoglobulins by conventional immunologic methods. The problems which have arisen can be briefly stated:

1. The low levels of immunoglobulins in intestinal secretions require concentration techniques in most cases.

2. The presence of proteolytic anzymes in intestinal secretions results in some degradation of immunoglobulins.

3. Antibody detection techniques sufficiently sensitive to detect antibodies present in low concentrations may not be applicable to intestinal juice, which for example, hemolyzes red cells in hemagglutination studies.

4. Quantitation of immunoglobulins by type (IgG, IgA & IgM) is complicated by the presence of globulin fragments which react with antisera against all the globulins.

Progress:

1. Ammonium sulfate precipitates of intestinal juice from normal Thai subjects were resuspended in normal saline, dialyzed and studied for immunoglobulin content. Immunoelectrophoresis shows the presence of IgA as the predominant globulin. A fast moving component is also noted, and reacts individually with all anti-IgG, IgA and IgM goat anti-human sera. The nature of this component is not clear at this time.

The most likely possibility is that it represents a proteolytic breakdown product common to all the immunoglobulins. Notably, the fast component may be absent, or occasionally multiple. This variability be related to how long the globulins have been exposed to enzyme action prior to aspiration of intestinal juice. Ouchterlony plate experiments indicate that the reaction with all antisera is due to the same fragment.

2. Quantitation of immunoglobulins has been performed by the precipitin ring technique (using antibody-agar plates) with purified immunoglobulins as reference standards. Table I shows the quantity of immunoglobulins in the proximal jejunal secretions of one normal Thai subject intubated six times on alternate days. It should be emphasized that the presence of a fragment reactions with all three antisera (see above) may seriously interfere with accurate quantitation of immunoglobulins by the precipitin ring method. The figures in Table 1, therefore, are strictly provisional and preliminary.

3. Search for antibody activities in specimens of intestinal juice from subjects convalescent with diarrhea has been undertaken. Microtitre Hemagglutination Technique. Antigens used in this technique were obtained by NaOH fractionations of all enteric bacteria isolated from the stool and intestinal secretions of each case of acute diarrhea. Since whole intestinal juice and ammonium sulfate globulin fractions of intestinal juice are hemolytic, bile free preparations of globulins were prepared by column chromatography using Sephadex G-25 and DEAE. The preparations obtained were not hemolytic, but contained immunoglobulins in quantities equvalent to whole intestinal juice. By hemagglutination testing, several subjects with shigellosis showed positive results, but the distribution of positive juices over the two week convalescent period was random and did not follow a clear-cut daily pattern. Sera from these patients showed a definite rise in antibody over the two week study period. Interpretation of results so far indicate that other factors in the intestinal juice, aside from its hemolytic properties, may interfere with the hemagglutination test as well.

Immunofluorescent staining-because of difficulties (described above) with the HA method, an indirect immunofluorescent staining technique has recently been used in attempts to demonstrate intestinal juice antibody. Data are not yet available regarding the value of this method.

Table I

Day of study	(mgm % intestinal juice)			Protein concentration intestinal juice (mgm%)	Specific Activity Immunoglobins (mgm Immunoglobins per mgm protein)			Ratios	
	IgG	IgA	IgM		IgG	IgA	IgM	IgA/IgG	IgA/IgM
1	29	26.5	7.2	305.4	.094	.087	.023	.91	3.68
3	21.5	31.0	10 0	277.7	.077	.112	.036	1.44	3.10
5	60	51	13.0	430.4	.139	.118	.030	.85	3.92
7	24	35	8.7	305.4	.078	.114	.028	1.45	4.02
9	21.5	41	13.0	333.2	.064	.123	.039	1.90	3.15
11	16	19	0	347.1	.046	.054		1.21	—

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Title: Study of Malabsorption. V. The Effect of Prophylactic Folic Acid In Preventing Tropical Malabsorption Syndromes.

Principal Investigator: Captain Andrew G. Plaut, MC

Associate Investigators: Captain Kenneth Blaydow, MC*
Lt CDR Gerald T. Keusch, USPHS
Captain Frank J. Troncale, MC

Period of Report: 1 October 1966-31 March 1967

OBJECTIVE: The purpose of this study is to evaluate the intestinul function of American Troops stationed in the tropics, and the value of folic acid in preventing intestinal illness.

DESCRIPTION: The study plan involves the performance of the following intestinal function tests in a group of soldiers stationed in Thailand.

- Lactose tolerance test.
- Xylose tolerance test.
- Neomycin-xylose test.
- Intestinal mucosal biopsy.
- Serum carotene and vitamin A, folic acid, total protein, protein electrophoresis, cholesterol, transaminase and alkaline phosphatase.
- Body weight.

After these baseline studies are completed the study group will divided into two groups, one receiving 5 mgm folic acid daily by mouth, and the other a daily placebo by mouth. Drug administration will be in a double-blind manner. During the period of drug administration, episodes of diarrhea or other intestinal illness will be investigated and recorded at the camp by the assigned Medical Corps Officer. Prior to leaving Thailand, members of the unit will be re-studied as described under baseline studies, and folic acid and placebo groups compared as regards intestinal function.

The study group is composed of 29 members of Company D, Special Forces. Lopburi, Thailand.

PROGRESS: Approximately 1 month following their arrived in Thailand 29 men were studied with the following results:

Lactose Tolerance Test

25 men were tested. 5 men had a flat lactose tolerance test (maximum blood glucose rise less than 20 mgm% followina lactose load) and 20 were normal. This corresponds to reports from the United States, where approximately 15-20% of normal persons also have flat lactose tolerance curves.

* Special Forces Physician

Mucosal Disaccharidase Assay

Data are not available at this time.

Xylose Tolerance Test

29 men were studied, and 27 were found normal regarding xylose absorption (greater than 5 gms xylose excreted in 5 hours after ingestion of a 25 gram dose). Of the two subjects who were abnormal, one was restudied a month later and found to have a normal test. The other subject was not restudied. Blood xylose determinations at two hours following ingestion of the sugar correlated fairly well with urine results.

Neomycin-Xylose "stress" Test

This involves the administration of Neomycin sulfate (two grams in divided doses) during the 24 hours preceding a xylose tolerance test*.

27 men were tested. Xylose malabsorption occurred in 12 men (less than 5 grams xylose excreted in 5 hours after ingestion of a 25 gram dose), and 5 men had borderline xylose absorption. Of the remaining 11 men, although their xylose absorption was in the normal range, most had less absorption than they had had prior to Neomycin administration.

Serum Carotene and Vitamin A

24 subjects were studied, and all were normal.

Serum cholesterol, transaminase and alkaline phosphatase were assayed in 24 subjects. Three individuals had slightly elevated alkaline phosphatase values, but other tests were normal.

Serum Total Protein, Globulin and Protein electrophoresis, among 27 individuals studied were all normal with one exception-this subject had a moderately elevated serum globulin level, predominately due to a rise in $\alpha 2$ globulin.

Following the baseline studies mentioned above the men were divided into two groups one receiving folic acid and the other placebo. The men will be re-studied prior to leaving Thailand.

* Recent experimental data in Thai subjects show that a small dose of Neomycin Sulfate will, within 24 hours, result in intestinal mucosal damage and xylose malabsorption. The significance of this phenomenon is currently under study to determine if it also occurs in non-Thai subjects. For this reason, the Neomycin-xylose test was performed with the American soldiers described here.

Title: Study of Acute Diarrhea in Thais

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Associate Investigators: Chanyo Benyajati, M.D.*
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Lt Cmdr Gerald T. Keusch, USPHS
Captain Richard C. Buchanan, MC

Period of Report: 1 April 1966-1 September 1966

Objective:

1. To define histologic changes in the upper small bowel during acute diarrhea.
2. To measure the degree of malabsorption occurring during acute diarrhea, and the extent of recovery 1-3 months later.

Methods:

Twenty-one adult male patients with acute diarrhea were admitted from the emergency room of Chulalongkorn Hospital to the renal-metabolic study ward of the same institution between 11 March and 29 June 1966. After an initial evaluation, the patients were kept at bed-rest, fed adlibitum, and started on supportive measures: intravenous fluids, and antispasmodic drugs if abdominal cramping was present. Antibiotics were not used, although many of the patients had taken them before admission. The mean duration of diarrhea after admission was 2 days. A bacteriologic diagnosis was made in 10 patients as follows: Shigella sp. 5, Salmonella sp. 4, and Vibrio cholera, El tor, 1. Stool examination for intestinal parasites was positive in 14 of the 21 patients (5 patients with two parasites): Hookworm 6, Entamoeba histolytica cysts 5, trophozoites 2, Trichuris 3, Ascaris 2, and Giardia 1.

The following studies were attempted during the first few days after admission: small intestine biopsy, 25 g xylose excretion test, Schilling test (vitamin B_{12} absorption), fecal fat analysis (during a daily dietary supplement of 75 g of butter fat), and gastric analysis after maximal histamine stimulation. On admission, blood was drawn for B-carotene, total cholesterol, albumin and globulin, and CBC. Eleven patients returned 4-11 weeks later for follow-up studies. Jejunal biopsies were carried out with a Crosby or a Carey capsule after x-ray verification of the location of the capsule. All specimens were fixed in 10% neutral buffered formalin and examined with a stereomicroscope, magnification x 20. Tissue was embedded in paraffin and sections cut at 7 microns. Hematoxylin and eosin and MacCallum-Goodpasture stains were done in all cases. Azure-eosin and PAS stains were done on selected material. All biopsies were examined without knowledge of the clinical situation and reviewed later with clinical information available.

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*** Chief, Pathology Dept., Ramathibodi Medical School

Results:

In 16 of the 21 patients an adequate jejunal biopsy was obtained during the acute illness. The villus pattern as observed through the dissecting microscope did not differ from the Thai norm. All specimens displayed to some degree the histologic abnormalities previously described in Thai people[1]. In addition, 6 of the 17 patients had considerable edema of the villus tips and polymorphomuclear leucocyte infiltration of the gut epithelium. No correlation could be shown between the presence of these acute changes and the type of agent demonstrated by bacteriologic methods.

Six of the patients had large numbers of eosinophils in the lamina propria. One of the six also h d acute inflammatory changes near the villus tips. All of these patients also had intestinal parasties. A comparison made after knowledge of the stool bacteriologic findings suggested that the intestinal lymphoid follicles were much more prominent in patients with Salmonella sp. than in those with Shigella sp. or, those in whom no isolation was made.

No bacteria were seen in the gut epithelium or lamina propria in any of the patients. Follow up biopsies from 4 patients failed to reveal significant changes when compared to their biopsies obtained during acute diarrhea.

The 5 hr. urine d xylose excretion in 20 patients during acute diarrhea averaged 4.56 ± 1.21 g. In all patients at follow up, the excretion was significantly higher, 6.06 ± 1.29 g. When the data from the 11 following up patients are paired with their values in the acute period, the excretion is likewise significantly higher (p 0.05).

Because of the difficulties of collecting a twenty-four hour urine specimen during acute diarrhea, the Schilling test was carried out satisfactorily in only 7 patients. In 4, excretion was below 7.5%, a value generally considered the lower limit of normal. Follow-up studies were carried out in two patients and were normal in both (34%, 21%).

Fecal fat excretion was measured in 15 patients acutely, and averaged 3.10 ± 2.16 g/day. Only one patient excreted over 5 g/day (7.7 g), the upper limit of normal. Follow up studies were carried out in 5 subjects, and averaged 1.89 ± 7.72 g/day. None were above 5 g/day. When the data from the 5 patients who were studied during both the acute and follow up period are paired, there is no significant difference between the two sets of observations.

Paired serum B carotene values were available from the acute (74.9 ± 35.8 μg%) and follow up (103.0 ± 27.7 ug%) periods in seven patients. This increase noted during follow up was not significantly higher. Paired serum cholesterol values in the same number of patients were, however, significantly greater (P 0.01) during the following up period (191 ± 27 mg%) when compared to the value obtained acutely (154 ± 21mg%). Serum albumin, available in acute and follow up patients in only 5 instances, averaged 3.33 ± 0.68 g% acutely and 4.31 ± .87 g% in the follow up period, a significantly higher value (P 0.01).

No difference was noted in paired values between mean serum globulin in the acute phase (2.57 ± 0.45 g%) and in the follow up period (2.45 ± 0.62 g%).

Hemoglobin was normal (over 13.0 g%) in all 21 patients acutely except in three who had values of 11.0, 12.5, and 12.6 g%. Follow-up values in the nine patients in whom hemoglobin was measured were all normal.

Gastric analysis following maximal histamine stimulation was carried out in twelve patients. In five, maximum acid production was 20 units or less in the acute period. Only one patient in the follow up period, originally with low acid production was re tested, and he was found to have normal acid production.

Discussion:

The dissecting microscope appearance of the jejunal mucosa was not strikingly different from that seen in normal Thais. No change was noted between acute and recovery biopsy specimens in patients so studied. The enteritis observed with the standard light microscope is qualitatively similar to that described earlier in human biopsy material. We were unable to establish morphologic criteria which could distinguish various etiologic agents. In contrast to various experimental diarrheas, we did not observe granulomatous lesions, epithelial ulceration or tissue invasion by bacteria. The absence of the latter, however, connot be established with assurance by the techniques employed. Flucrescent antibody techniques might well be more fruitful.

Moderate impairment of xylose absorption was noted in many of the patients during the acute period of diarrhea however, all had returned to normal at the time of follow up. This finding confirms other work on actue diarrhea done in East Pakistan, with the exception that persistent xylose malabsorption was noted in a few of the patients in the latter study. Vitamin B absorption, impaired in 4 out of 7 patients in the acute phase of diarrhea, is generally considered to be a reflection of ileal absorptive capacity. Since most bacterial diarrheas (cholera excepted) are thought to affect the terminal ileum and colon, it is not surprising that impaired Vitamin B absorption occurred with such frequency. Fecal fat excretion was normal in all but one of 15 patients tested during acute diarrhea, and was likewise normal in all 5 tested at follow up. This test is a rather crude measure of intestinal function, and becomes impaired only when major gastro intestinal disease processes, such as celiac sprue or cystic fibrosis of the pancreas, are present. The absence of steatorrhea in acute diarrhea is thus consistent with the mild nature of the disease in our patients.

Low gastric acid production in acute diarrhea is previously unreported. Unfortunately, follow up studies were not available to find out if gastric secretion returned to normal. The validity of this observation, furthermore, is lessened by the fact that fluoroscopic placement of the nasogastric tube to ensure complete collections was not done, and that small amounts of blood were present in some specimens, which might also account for a lowered acid production. Nevertheless, the observation is quite interesting since gastric acid is known to be a major bactericidal defense mechanism and if achlorhydria can be shown to exist before diarrhea occurs it, may point to a new mechanism in the pathogenesis of bacterial diarrhea.

Title: Studies on the Nonspecific Jejunal Abnormality of Thai People

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Associate Investigators: Lt. CDR Gerald T. Keusch, USPHS
Captain Louis H. Miller, MC
Lt. Colonel Ray A. Olsson, MC
Colonel Edward H. Johnston, MC

Period of Report: 1 November 1965 31 December 1966

Introduction: It has been implied that tropical sprue is widespread as a subclinical entity in hot climates (1), Jejunal biopsy of asymptomatic adults in India (2), Pakistan (3, 4), and Thailand (5) has shown a histologic lesion of varying severity, consisting of focal villous thickening and fusion with cellular infiltration of the lamina propria. Under the dissecting microscope this abnormality is associated with leaf shaped or ridged villi, and in more extreme cases, convolutions. Abnormalities of xylose absorption have been noted in a significant percentage of such asymptomatic individuals (1 4). Recently, Klipstein et al. (6) were able to diagnose tropical sprue in Haiti, although it was previously unknown there, by selecting patients with sympt suggestive of the disease. In addition, the ease with which the same anatomic and biochemical abnormalities were found in a group of asymptomatic Haitians led these workers to speculate that sprue was a disease with a wide range of expression, with clinical cases representing but a fraction of the total number affected.

Because a jejunal lesion has been found commonly in asymptomatic subjects in Thailand (5), we undertook to study its functional expression with a wider variety of tests than previously employed, to assess the lesion as a possible precursor of tropical sprue.

Methods:

Forty volunteer subjects, primarily from two villages on the outskirts of Bangkok, gave informed consent and were hospitalized for the studies to be described. There were 22 females, age 16 72 years and 18 males, age 13 45 years. History and physical examination were within normal limits in those accepted for the study. The following were also performed in every subject: WBC, Total serum protein and electrophoresis, serum phosphorus, calcium, iron, B-carotene, hemoglobin, and hematocrit. Urinalysis, blood urea nitrogen and serum creatinine were carried out in all subjects and found to be normal by North American standards. Chest X rays, performed in all subjects, were also normal.

Five hour urinary d xylose excretion and 2 hour serum xylose levels were measured in fasting subjects. All subjects received 25 g. of xylose except for three who received 0.5 mg/kg (13.7, 22.0, and 22.6 g.).

Urine and serum specimens were kept frozen after collection. The xylose determination (7) was performed within 24 48 hours after completion of the test.

Vitamin B_{12} absorption was measured by the Schilling technique (8) using Co^{57} Vitamin B_{12}* with Intrinsic Factor. Vitamin A absorption was tested in fasting subjects by the oral administration of a dose of 260,000 international units mixed in corn oil. Serum levels were measured before and five hours after ingestion of Vitamin A (9).

Lactose tolerance tests were performed using a dose of 1.5 g/kg body weight. Blood glucose was measured (10) fasting and at 30 and 60 minutes in all subjects, and generally at 15, 45, 90, and 120 minutes as well.

Glucose absorption was tested using an oral dose of 0.75 g/kg body weight, equivalent to the glucose content of lactose used above. A standard oral glucose tolerance test (100 g. dose) was also done in 4 subjects. Blood was taken for sugar determinations at the same time intervals as for the lactose tolerance test.

Stool fat analysis by the method of van de Kamer (11) was carried out on 3-to 6-day collections. Because fat intake in the average Thai diet is low (12), a daily supplement of 7.5 g. of butter was given during the collection period. Carmine red was used to make the beginning and end of the collection periods.

Fluoroscopic examination of the upper gastrointestinal tract with small bowel follow-through studies were also performed +.

Small bowel biopsy specimens were taken from the jejunum with either a Crosby (13) or a Carey (14) biopsy capsule after x-ray verification of its position.

Results:

Laboratory data are shown in Table 1. Urinary excretion of d-xylose following a 25 g. dose was 5.84 ± 1.54 g. (S.D.) of the administered dose in 36 subjects. The mean two hour serum level was 39.8 ± 7.5 mg/100 ml. (S.D.). The percentage of xylose excreted by the 3 subjects given the smaller dose (0.5 g/kg body weight) was comparable to that following a 25 g. dose. Mild diarrhea consisting of 2–4 watery stools occurred after ingestion of the 25 g. dose in 3 subjects. Five hour urine volumes were above 225 ml in all subjects tested.

Little or no increase in blood glucose occurred following oral lactose in any of the 39 subjects tested (Fig. 1), the mean rise being 2.97 ± 3.07 mg/100 ml. Diarrhea, consisting of up to 10 loose stools, and/or abdominal cramps, occurred in 28 of the 39 subjects.

In contrast to the flat lactose tolerance test, glucose absorption was normal. The mean maximum rise in blood sugar after ingestion of 0.75 g/kg body weight glucose was 42.5 ± 20.5 mg% in 33 subjects (Fig. 1). Only four subjects showed a rise of less than 20 mg% (7, 15, 16, and 19 mg%). In 4 other subjects given 100 g. of glucose the mean maximum rise in blood sugar was 61.5 mg%.

Vitamin B_{12} absorption was normal in 33 of 34 subjects tested (Fig. 2). Only one subject, with a hemoglobin of 12.4 g. was abnormal (4% excretion).

Stool fat excretion averaged 2.3 ± 1.4 g/day in twenty-nine subjects tested. The values were below 5 gms. in all except two in whom the fat excretion was 6.3 and 6.8 g/day (Fig. 2).

Vitamin A tolerance in twenty-four or twenty-seven subjects showed a rise of more than 125 mg% above baseline (15).

The mean serum total cholesterol was 177.5 ± 31 mg/100 ml. There was no correlation between xylose excretion and serum cholesterol (16).

*Racobalamin, Abbot Laboratories.

+ We are indebted to Dr. Chitti Palavatana, Maj., RTA, for performing the x-ray studies.

The following mean serum levels ([illegible]) were obtained (the number of subjects tested is in parenthesis): Calcium, 9.31 ± 0.49 mg/ml (29), cholesterol, 177.5 ± 31 mg/100 ml (25), beta carotene, 133.1 ± 65.0 ug/100 (34), albumin, 4.18 ± 0.56 g/100 ml (32). Hematologic findings were as follows: males, hemoglobin 13.8 ± 1.9 g/100 ml (18), females, hemoglobin 12.1 ± 1.2 g/100 ml (22).

Fifteen subjects were found to harbor the following small intestine parasites: Ascaris alone in eleven, Ascaris and Hookworm in one, Hookworm alone in one, Hookworm and Strongyloides in one, and Giardia in one. No difference in absorption tests were noted between these subjects and the twenty four whose stools were negative for parasites, although it was interesting to note that the one Giardia-positive stool was found in subject No. 8, whose xylose excretion 2.99 g, was the lowest recorded. The small bowel biopsy did not show the protozoa in this subject, and unfortunately, duodenal aspiration was not performed.

No abnormality was seen in the x-ray of the small bowel in the 29 subjects studied except for mild mucosal irregularity in the ileum of one. In six subjects, worm like objects, probably ascarids, were seen in the mid ileum.

One duodenal and thirty nine jejunal biopsy specimens were obtained from the forty subjects. The biopsies showed the same mild abnormalities including focal fusion or blunting of villi and increased cellularity of the lamina propria as previously reported from Thailand (5). Under the dissecting microscope the villi were broad and leaf shaped, with occasional biopsies showing ridges or convolutions. In no specimen did finger like villi predominate, and none of the biopsies were flat. The single duodenal biopsy was very similar in appearance to the specimens from jejunum except for the presence of Brunner's glands. No correlation was noted between the degree of microscopic or gross appearance of the biopsies and any of the above absorption tests, x-rays, or the presence or absence of parasites.

Discussion

In this population of asymptomatic Thai subjects, absorption of fat, vitamin B_{12}, vitamin A, and glucose was normal in comparison to North American subjects (17). Xylose and lactose absorption and the histologic appearance of the jejunal mucosa were different. Since changes of similar degree have been found by others in healthy subjects and interpreted as indicators of subtle intestinal disease (2-6), it is important to ask whether these findings, in the absence of malabsorption of nutritionally important substances, acturally represents a disease.

Urinary xylose excretion following a 25 gm dose in this population (5.84 ± 1.54 g) was the same as Gardner and Perez-Santiago (5.60 ± 0.60 g) and Butterworth, et al. (18) (5.70 ± 1.40 g) have reported from Puerto Rico, and Lindenbaum et al. (4) from East Pakistan (5.38 ± 1.75 g) with the exception of one study (19), these results are definitely lower than other series of Americans, whether studied in the United States (20, 21), Thailand (22), or the "protected" Westerners referred to by Lindenbaum et al. (4) in Pakistan, where the lower limit of normal xylose excretion is 5 g.

The xylose excretion in the present study and in a group of Pakistanis reported recently in another study by Lindenbaum et al. (23) appear normally distributed. When this occurs, indicating a homogeneous population, it is not valid to impose a criterion of normality (e.g. greater than 5 g excretion) derived from a different group (North Americans) which arbitrarily splits the population into two nearly equal segments. While the reasons for this mild reduction in xylose excretion is not clear, it is important to piont out that these are clearly different than values found in classical sprue (17). The absence of steatorrhea or vitamin B_{12} malabsorption in the Thai population supports the concept that this is not tropical sprue. In addition there was no correlation between xylose excretion and serum cholesterol, as recently found in Puerto Rico (16).

The lactose tolerance test was abnormal in all subjects. Although it has been observed in tropical sprue (24), lactose malabsorption has also been described in a variety of other gastrointestinal diseases (25), as a possible genetically controlled enzyme defect (26), and in a surprisingly high percentage of normal adults (27). In view of the apparent universal occurrence of lactose malabsorption in Thais, the test is of no value as a screening test for tropical sprue.

The interpretation of the non specific jejunal abnormality is difficult because other factors must be considered inits pathogenesis. Similar histologic changes have been reported in kwashiorkor (28), hookworm disease (29), some viral illnesses (30), rosacea (31), in acute diarrhea (32), and after MER 29 (Triparand) Administration (33). None of these conditions were operative in our subjects, although it does emphasize that the intestine has limited ability to respond to injury. What, then, is the relationship between this non-specific histologic abnormality and tropical sprue? In has been suggested that there is a spectrum of small bowel disease in the tropics which ranges from mild and asymptomatic malabsorption to overt sprue (6). Furthermore, this coexistence of tropical sprue and milder forms of jejunal abnormality in the same tropical population has been cited as evidence supporting an association between the two processes. However, intestinal function in Thai subjects in the present study with non-specific jejunal abnormality is different than that in overt tropical sprue, and further, clinical tropical sprue appears to be rare in Thailand. Thus, we have no evidence that there is any relationship between these two conditions. A recent study of Pakistanis (23) with similar histologic changes failed to show improvement in absorption tests after treatment with tetracycline or folic acid, which further indicates that the mild non specific jejunal biopsy changes, so common in tropical areas, are not necessarily of tropical sprue.

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STUDY REPORTS

1. Title: Enzyme Histochemical Studies of the Non-Specific Jejunal Lesion in Thai People

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Associate Investigators: Gerald T. Keusch, LCDR, USPHS
Frank J. Troncale, Caption, MC, USA

Period of Report: 1 May 1966 31 March 1967

Objective:

To evaluate: 1. The enzymatic staining actiaity of the mucosa of the small intestine of the Thai people. This would show whether the mucosa with "mild jejunitis" or non specific abnormalities displays any enzymatic aberration. It would also help establish a base line for further comparison with the staining pattern seen in other enteric diseases.

2. Enzymatic pattern in normal subjects after manipulation of the intestinal tracts with some drugs like antibiotics.

3. Enzymatic staining pattern in various enteric conditions.

Description:

Biopsies were obtained from the mucosa of the proximal jejunum with the Crosby-Kugler biopsy capsule. Subjects were fed a high fat diet by the addition of 75 gm of butter per day. All subjects fasted for 10-14 hours before biopsy. The tissue was divided and one piece was fixed in 10% buffered neutral formalin for gross and microscopic examination. The other piece was oriented villus side up on a block of fixed liver or kidney tissue and quick frozen sectioning at 20°C. Eight micron sections were picked up on cover slips, air dried, and stained on the same day. In a few cases sections were stored in an air-tight container at 70°C and stained on the following day. Tissue was examined for the activities of alkaline phosphatase, acid phosphatase, non specific esterase, succinate dehydrogenase, DPN diaphorase, and glucose-6-phosphate dehydrogenase. Unstained cryostat sections were fixed in formalin, and stained with oil-red 0 and hematoxylin. Controls for the enzymatic staining were based on the following procedures: 1. Simultaneous incubation of fresh frozen sections of mouse liver and intestine with the sections of human intestine. 2. Incubation of sections in media without specific substrate. 3. Simultaneous incubation of sections from different cases. 4. The use of specific inhibitors as described for the various enzymes. The activities of the euzymes were graded ronghly 0 to 4+, according to the overall intensity of staining.

Progress:

One hundred and four biopsies obtained from three groups of patients were studied:

1. Thirty-nine biopsies from twenty-eight Thai adults with normal intestinal absorption of xylose and fat.

2. Sixty biopsies from the above subjects receiving neomycin and biopsied before, during and after the drug.

3. Five biopsies from one Thai patient with tropical sprue.

Results:

Enzymatic staining activities.

Group I. All subjects in this group showed histological evidence of "Nonspecific jejunal abnormality". The enzymatic staining reactions were similar to the pattern seen in the mucosa of normal North American controls. Alkaline phosphatase stained strongly in the brush border of the absorptive columnar epithelial cells of the villi, ending abruptly at the level of the crypt. Activity was also observed in occasional blood vessels in the lamina propria. The activity of ATPase was strong in the brush border of the absorptive cells of the villi and was not present in the crypt. A small amount of reaction product was observed in the apical part of the cytoplasm of absorptive cells and also in occasional vessels in the lamina propria and muscullaris mucosa. Acid phosphatase activity was localized to the supranuclear part of the absorptive epithelial cells, as well as in macrophages within the lamina propria. Faint reaction was observed in crypt cells. Strong activity was noted in Paneth cells at the bottom of the crypt. The activities of succinate dehydrogenase, glucose-6-phosphate dehydrogenase and DPN diaphorase were prominent in the peripheral part of the cytoplasm of the absorptive epithelial cells of the villi, particularly in the apical and supranuclear portion of the cells. Activity in the crypt was much less intense. Esterase activity was localized mainly to the supranuclear portion of absorptive epithelial cells. Lesser activity was noted in crypt cells. Esterase activity was also observed in occasional macrophages.

The histochemical staining pattern was consistent in slender finger villi. Occasionally patchy areas of minimally decreased activity (3+) for ATPase, succinic and G-6-P dehydrogenase were observed in short and blunted villi. This might represent sectioning along the long axis of a leaf or ridge shaped villus. Acid phosphatase granules in the supranuclear portion of the absorptive cells in Thai intestinal mucosa appeared to be more abundant than in comparable specimens from American subjects studied in our laboratory. Acid phosphatase activity in the lamina propria was also more prominent in the Thai specimens because of the presence of greater numbers of macrophages.

Group II. Fourteen subjects received neomycin orally (2-8 gm/day). In ten subjects neomycin was given for 7 days and in 2 subjects for 3 days. In two subjects a single 2 gm dose of neomycin was given orally, and biopsy performed 6 hours later and again at 28 hours. All patients showed biochemical evidence of malabsorption, including the two cases given a single dose of neomycin. These abnormalities returned to norm[illegible] several days when the drug was stopped. In four out of subjects receiving a 7 day course of neo[illegible] gm/day, patchy areas of decreased staining (2 3+) in the activities of ATPase, alkaline pho[illegible] and succinic dehydrogenase. This was accompanied by a slight increase in the activity of acid phosp[illegible] in the macrophages and in occasional absorptive opithelial cells. In one subject given 8 gm of neomycin daily, slight decrease (3+) in the activities for ATPase, alkaline phosphatase, and succinic dehydrogenase was noted after 3 days of drug administration, while at 7 days activity was markedly decreased (1 to 2+). Acid phosphatase and esterase activities in macrophages and occasional absorptive epithelial cells were slightly increased. In two cases given a single dose of drug, a decrease in the staining activities of all enzymes at 6 hours was observed. Twenty-eight hours after neomycin the staining activity in both cases returned to normal. In two cases, one biopsied at 5½ hours, and the other at 24 hours after 2 gm of neomycin, slight irregularity in staining activity of ATPase was observed while succinic dehydrogenase activity was markedly reduced. The activity of acid phosphatase was stronger than usual in

one case while in the other it was within normal limits. At 3 days of drug administration, the succinic dehydrogenase activity remains weak. The enzyme staining pattern returned to normal 24 hours after the drug administration was discontinued.

Group III. One Thai patient with the clinical and biochemical diagnosis of tropical sprue was studied serially. Histologic features of the jejunal biopsy revealed marked villus shortening, epithelial atypism and a plasma cell infiltrate in the lamina propria consistent with the diagnosis of tropical sprue. There was a marked decrease in the area of staining of all enzymes secondary to the loss of villus surface. The intensity of staining for succinic dehydrogenase was markedly reduced (O 1+) while ATPase was slightly decreased (2 3+) in places. The intensity of staining for acid phosphatase activity was strong in macrophages and the absorptive epithelial cells on the surface of the flattened villi. One month after initiation of folic acid therapy there was definite increase in the area of staining for ATPase and alkaline phosphatase as regeneration of villi occurred. Succinic dehydrogenase activity did not improve until two months after treatment. By three months the enzyme staining was within normal limits for Thai subjects, while the villi were still relatively short. At four months the biopsy was normal.

Lipid staining.

After 12-14 hours of fasting, 10 out of 12 biopsies from subjects in group I eating the usual low fat Thai diet, showed no visible oil red O positive droplets either in epithelial cells or in the lamina propria. The remaining 2 biopsies showed occasional but rare droplets in macrophages in the lamina propria. In contrast normal North Americans subjects on a high fat diet ordinarily have fat droplets in the lamina propria. Therefore our subjects were given a high fat diet on the day prior to biopsy. Under these conditions the results were comparable to normal American subjects. In group II, four patients with neomycin induced steatorrhea had fat droplets within the absorptive epithelial cells at the tip of the villi. as well as in the upper part of the lamina propria. (When of these subjects was biopsied without fat supplementation in the diet, no fat was visible in the specimen). The patient with tropical sprue had small and large fat droplets in both the apical and subnuclear portion of the cytoplasm and in the region of the basement membrane.

Discussion:

Jejunal abnormalities in "normal" Thai small intestinal mucosa were first described by Sprinz et al. They found evidences of malabsorption based on a 5 gm D-xylose absorption test and serum carotene level and suggested that the morphological findings were compatible with early sprue. A recent study by Troncale and associates, however, showed that low income, asymptomatic Thai subjects with non-specific abnormalities in the small bowel had normal xylose excretion with the 25 gm test dose, normal vitamin B^{12} and vitamin A absorption and normal fecal fat content on a high fat diet. They concluded that the jejunal lesion could not be considered an early sprue lesion. The group of patients reported here, from the same population studied by Troncale et al, showed normal enzyme histochemical staining patterns and supports this conclusion.

Two factors appear to be critical in absorption or transport through the intestinal wall, the total surface area available for absorption and the functional efficiency and integrity of the individual absorptive unit. It has been pointed out that the total surface area as well as the number of absorptive epithelial cells would be significantly decreased as the villus shape changes from finger to leaf and to convolutions. Creamer has concluded that the leaf villus has 50% of the surface area of the finger villus while a convoluted surface is reduced to 25%. Histochemical staining by distinguishing mature epithelial cells from young crypt cells may assist in the evaluation of the functional integrity of these cells. Histochemical staining also clearly reveals the separation between crypt and villus and thus directly displays the absorptive surface.

In the present study neomycin, known to cause malabsorption in normal subjects, was used to study the response of the Thai with non specific jejunal abnormalities. Changes in the histochemical staining pattern

correlated with histologic evidence of cellular injury. These included cellular atypism and increased numbers of mitotic figures and goblet cells. Because. the histochemical techniques used are qualitative, actual correlation of enzyme activity with the dose of neomycin was not possible. Kent, et al have shown acute changes in enzyme histochemical staining of the small intestine from staphylococcal enterotoxin in monkeys with recovery in 24 hours. Similar acute changes have not been previously described in man. In the present study alterations in enzyme staining occurred after a single dose of neomycin. What is of greater interest is that some degree of histochemical and histologic recovery following acute neomycin injury occurred even when administration of the drug was continued. Function, however, did not improve. Although actual enzyme activity may still have been considerably depressed from control in this situation and not revealed by the staining techniques, some adaptation appeared to take place. The nature of this is not kown. The lack of correlation between cellular injury as revealed by light microscopy or histochemical study and the clinical evaluation of intestinal function in tropical sprue and in the neomycin lesion may be a result of the insensitivity of the clinical methods in use.

It is generally accepted that the morphologic response of the small intestinal mucosa to injury is somewhat limited and nanspecific, regardless of the type or nature of the stimulus. Enzyme and lipid histochemical staining adds another dimension to morphologic observations. However, many problems remain to be clarified. What is the meaning of differences in degree of enzymatic staining? Why is there such poor correlation between the pattern of enzymatic staining or morphology and intestinal function? Little information is available on sequential changes in both enzyme and lipid staining in physiological as well as disease states in man. These changes may occur in hours rather than days. Furthermore, the injury due to neomycin is quickly repaired, whereas the sprue lesion is not. The effects of chronicity of the injurious factor remain to be elucidated.

Summary:

Thai subjects with non-specific jejunal abnormalities have normal mucosal enzyme activity and pattern of lipid distribution.

Administration of neomycin results in decreased activity of ATPase and succinic dehydrogenase. Lipid droplets accumulate within epithelial cells in neomycin induced steatorrhea. Histochemical staining correlates well with visible epithelial cell damage but not with absorptive function.

Title: Neomycin Enteropaihy and Malabsorption.

Principal Investigator: Lt. CDR Gerald T. Keusch, USPHS

Associate Investigators: Captain Richard D. Buchanan, MC
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Period of Report: 1 April 1966 31 March 1967

Objectives:

Neomycin, an aminoglycoside antibioitc, causes a reversible malabsorption syndrome in man. Faloon and colleagues have demonstrated increased facal excretion of fat, nitrogen, and sodium and potassium and decreased serum cholesterol as well as malabsorption of B-carotene, vitamin B , d-xylose, iron and I^{131} labelled trioleate, due to oral neomycin administration. These workers have reported changes in the jejunal mucosa consisting of clubbing of the villi and edema and round cell infiltration of the lamina propria due to the drug. Hvidt and Kjeldsen reported malabsorption of both fat and d-xylose on 3 gm. per day of neomycin, and Samuel and Steiner showed the cholesterol lowering effect to occur with as little as 0.5 gm of neomycin daily.

The pathogenesis of the syndrome is not known. Gluten restriction or steroid administration may or may not effect neomycin-induced steatorrhea. Recently attention has been focused on alterations in bile salt metabolism due to neomycin which may effect mucosal function or intraluminal hydrolysis of fat. The histologic changes reported by Jacobson et al and thought to result in "physical blockage" of absorption are disputed by Rubin, and may not correlate with changes in function.

The present study was undertaken to evaluate the acute effects of neomycin and to study the evolution of functional and structural changes.

Meterial and Methods:

Thirty normal Thai adults, 27 females and 3 males gave informed consent for the studies to be described. The mean age was 25.3 (16-47) years. All were hospitalized and studied before, during and after drug administration. Neomycin was given orally as the sulfate, 0.5 gm tablets equivalent to 0.35 gm neomycin base.

Effects of neomycin were assessed with the following:

1. Twenty-five gram xylose tolerance test, with measurement of 5 hour urinary excretion and 2 hour serum xylose by the method of Roe and Rice.

2. Sucrose tolerance test with measurement of blood total reducing substance by a modification of the method of Hoffman adapteu to the autoanalyzer before and 30, 45, and 90 minutes following oral administration of 1.5 g/KBW sucrose.

3. Fecal fat content, determined by the method of Van de Kamer et al in 3-5 day collections marked with carmine-red dye. A daily supplement of 75 grams of fat in the form of butter was given during collection periods.

In 5 subjects Vit B_{12} absorption was tested by the Shilling technique using Co^{57} labelled vitamin* with exogenous intrinsic factor.

In 6 cases intestinal water absorption was assessed by administration of an oral water load of 20 cc of water/KBW. All urine was collected and volume and osmolarity (Fiske Osmometer) were measured at 60, 80, 95, 110, 125, and 140 minutes. Maximum water diuresis was maintained by oral water replacement equal to the urine volume at the end of each collection period. Osmolar clearance (Cosm) and free water clearance (C_{H_2O}) were calculated. The collection period during which maximum diuresis was obtained was noted and the end of that collection period recorded as the time to reach maximum C_{H_2O}.

Biopsy specimens were obtained with the Crosby-Kugler capsule from the region of the ligament of Treitz, oriented on filter paper, and divided. One section of tissue was fixed in 10% buffered neutral formalin for histologic examination. The second portion of the specimen was oriented villus-side up on a block of kidney tissue and quick frozen with liquid nitrogen or dry ice. Sections were cut at 5° to 15°C add examined for the activities of adenosine triphosphatase, alkaline phosphatase, glucose-6-phosphate dehydrogenase, non-specific esterase, and succinic dehydrogenase. In most cases sections were processed within 4 hours of biopsy. In a few cases tissue was kept for 24 hours in a freezer at -70°C before staining. Sections from normal Thai and mouse intestine served as positive controls. Addition of inhibitor substances or substrate withdrawal established negative controls. Sections were post-fixed in formalin and stained with Oil-Red-O.

In 4 cases, jejunal tissue was homogenized in 1 cc of saline and cultured on blood agar pour plates for total aerobes and anerobes. Classification of organisms was made on the basis of colonial characteristics and microscopic morphology of gram stained smears. Growth of *Cl. perfringens* insured that strict anerobiasis was maintained.

Results:

Xylose Absorption: Five hour urinary xylose excretion decreased in all subjects given neomycin (Table I) from the mean control value of 6.26 ± 1.60* gm to 2.01 ± 0.75 gm ($p < .01$). Recovery was rapid and by 1 week following cessation of drug administration the mean xylose excretion was 5.64 ± 1.76 gm.

The 2 hour serum xylose concentration paralleled urinary excretion. The mean control value of 39.60 ± 7.0 mg per 100 ml decreased to 17.44 ± 3.83 mg per 100 ml during the drug period. The 2 hour serum level was 34.70 ± 5.92 mg per 100 ml within 1 week of stopping the drug.

Xylose tolerance tests were begun within 6 hours of a single 2 gram dose of neomycin in 6 subjects. An additional 5 out-patients were similarly tested. Xylose excretion decreased in 10 of the 11 subjects and the 2 hour serum level was depressed in 9 of the 10 subjects in whom this measurement was obtained (Table II). These changes were highly significant ($p < .01$) for both urine and serum determinations.

Xylose absorption was tested on alternate days in 5 subjects given increasing doses of neomycin (Fig 1,2). In 4 of the 5, xylose was malabsorbed when first tested after 24 hours on drug. The remaining subject (CH) malabsorbed xylose on study day 4 after receiving neomycin for 3 days. This subject did not

* Racobalmin, Abbott Laboratories

* All data expressed as mean ± 1 S.D.

manifest other stigmata of neomycin malabsorption, i.e. steatorrhea or sucrose malabsorption. Changes in the 2 hour serum xylose concentration (Fig 2) were a less sensitive indicator of neomycin induced malabsorption. There was little apparent effect from increasing the dose of neomycin once xylose malabsorption was present.

Sucrose absorption: The mean maximal rise in blood glucose following an oral sucrose load in 10 subjects was 59 ± 12.4 mg per 100 ml (Fig 3). During neomycin administration this decreased to 24.5 ± 10 mg per 100 ml ($p < .01$). In 9 of the 10 subjects the maximal rise in blood glucose during drug administration was less than 20 mg per 100 ml. When neomycin was discontinued the mean maximal rise was 69.5 ± 17.1 mg per 100 ml. Recovery was rapid and values returned to control within 1-2 days of discontinuing neomycin.

In 7 patients a sucrose tolerance test was performed within 6 hours of a single 2 gm dose of neomycin (Table III). Three of the 7 showed a "flat" response, and the blood sugar rise was blunted in the remaining 4.

Fecal Fat Excretion: Twelve of 15 subjects given neomycin excreted increased fat in the stool, including one subject taking only 1 gm per day of neomycin (Fig 4). In the entire group mean fecal fat content during the control period was 1.8 ± 1.2 gm per day. This increased to 5.0 ± 3.0 gm per day during neomycin administration. The post neomycin value was 1.8 ± 1.1 gm per day.

Vitamin B_{12} Absorption: In 5 subjects studied during control and neomycin periods, 3 showed impaired vitamin B_{12} absorption during drug administration, including 1 subject given only 1 gm per day of drug. In the remaining 2 subjects no significant change occured.

Water Load Tests: Water load data are summarized in Table IV. Although the maximum C_{H_2O} decreased during neomycin administration in every case, there was no detectable delay in the time to maximum diuresis.

Bacteriologic Culture of Jejunal Tissue: Small numbers of streptococci or staphylococci were found which diminished somewhat after 7 days of drug administration. No new organisms were found during or after drug except in one post neomycin biopsy (SW) from which a gram-negative, anerobic fusiform rod was recovered.

Histologic Study: Pretreatment Biopsies: The surface pattern under the dissecting microscope was regular, with tall leaf shaped villi the predominant form present. There were occasional tongue shaped villi. Finger-like forms were rarely seen. Sections showed nonspecific changes in varying degrees as previously described in normal Thais.

Neomycin Treatment Biopsies: 6 hour Biopsies: There was no change in appearance under the dissecting microscope. In sections, there was some shortening and broadening of the villi. The vascular loops were usually inconspicuous. Polymorphonuclear leucocytes and lymphocytes in small numbers were present in the epithelial layer. The columnar epithelial cells appeared shrunken. Many nuclear fragments were present in the epithelial cells. These changes were more marked near the villus tip. (The changes are similar to those shown in the report on Paramomycin effects on gut structure and function).

24 hour Biopsies: There were no uniform changes in the dissecting microscope appearance. A few specimens were coated with thick opaque mucus. The changes in the epithelial cells were similar to those seen at 6 hours. Mitotic figures appeared more abundant than in the 6 hour material.

72 hour Biopsies: Most of the specimens were covered with heavy mucus containing particulate debris. In comparison to the previous specimens the epithelial cells were more normal in appearance. Most villi contained prominent vascular loops. Mitoses were frequent. Goblet cells were irregularly distributed.

Increased numbers of plasma cells were present in the lamina propria. Many macrophages containing nuclear debris and bacteria-like objects were present near the villus tips. The latter particles, variable in shape, ranged in size from 2.5 microns to the lower limit of resolution and were visible in biopsy material fixed in neutral 10% formalin, Zenker's (AFIP) and Bouin's fluids, although less sharply defined in the latter fixative. They were intensely basophilic with alum hematoxylin, PAS negative, stained red with Brown-Brenn and MacCallum-Goodpasture stains, and were metachromatic with Giemsa and azure-eosin stains. The Feulgen reaction was positive with only a portion of this material.

Post-Drug Biopsies: Biopsies secured from 5 to 14 days after cessation of neomycin could be distinguished from control only by the presence of mononuclear phagocytes containing particulate debris. These progressively diminished in number and were usually not detectable by the end of the second week following the drug.

Enzyme Histochemistry: The pattern of enzymatic staining in the control specimens was normal. Neomycin administration resulted in patchy abnormalities in staining for ATP'ase, alkaline phosphatase, and succinic dehydrogenase (to be reported in detail elsewhere). In 2 subjects studied at 6, 24, and 72 hours on neomycin, 4 gm per day, there were more profound abnormalities in the 6 and 24 hour specimens than in the 72 hour specimen.

In 4 patients with neomycin-induced steatorrhea, fat droplets were present within epithelial cells as well as in the upper portion of the lamina propria 12 hours after a high-fat meal.

COMMENTS

The present studies of neomycin-induced malabsorption differ in two respects from previous reports. Firstly, the acute effects of the drug were evaluated, and secondly the subjects were Thai people whose jejunal mucosa is histologically different than normal Americans. The fact that neomycin administration can lead to histologic, histochemical and functional changes within 6 hours of a therapeutic dose is previously undescribed.

The question naturally arises whether or not this extreme sensitivity of the Thai small bowel is a consequence of pre-existing damage represented by non-specific jejunal abnormalities. Function studies in Thais, however, have failed to reveal significant abnormalities. When Americans are studied after only 24 hours on a small dose of neomycin the d-xylose test, including both urinary excretion and the 2 hour serum level is significantly depressed from control (unpublished data). Rogers et al administered high doses of neomycin to Americans and described epithelial cell changes including flattening, vacuolization, and loss of nuclear polarity, as well as macrophages in the lamina propria containing "ingested cellular fragments" after 1-2 weeks of drug. It is not certain if the latter observation is similar to our findings in Thais. It appears, however, that the response to the drug in Americans both in terms of function and structure may be qualitatively, if not quantitatively, similar to Thais.

The pathogenesis of neomycin-induced malabsorption is not known. Jacobson et al suggested that inflammatory changes in the jejunum were responsible for "physical blockage of absorption". These histologic changes have been the subject of controversy and are disputed by some authorities. The features described by Jacobson et al as typical for neomycin enteropathy resemble the usual Thai bowel in whom there is no accompanying malabsorption. When neomycin is given to Thai subjects the most striking early abnormality is in the absorptive epithelial cell where nuclear and cytoplasmic changes occur within 6 hours of the first dose of drug. Accompanying this are histochemical abnormalities and malabsorption of xylose and sucrose. It is clear that the absorptive cell itself is being affected by neomycin, and one need not implicate changes in villus shape or in the degree of round cell infiltration in the lamina propria to account for functional abnormalities. While some histologic and histochemical improvement in the appearance of the epithelium

appears with continued administration of drug, other evidence of continued injury is present, e.g. mucus discharge, increased numbers of mitoses in the crypts, and abnormal function. It may be that neomycin causes generalized poisoning of the epithelial cells acutely and a more specific biochemical lesion with chronic administration.

The nature of this lesion is open to speculation. The mechanism of action of neomycin in bacterial systems Is to interfere with ribosomal reading of m-RNA information in protein synthesis. The result of this is synthesis of inefficient or inactive protein. Each absorptive function studied by us probably relies upon an intermediary protein for hydrolysis and/or absorption. Sucrose for example, is not absorbed unless enzymatically split into its component monosaccharides which are then actively transported across the intestinal wall. Xylose transport is mediated by a carrier molecule, and some evidence suggests this is a protein. Fat, once within the cell as triglyceride, required attachment to protein to form chylomicrons in order to escape the cell. Evidence that these proteins in man are affected by neomycin includes disaccharidase assay, histochemical evidence of depressed enzyme activity, and demonstration of fat within the epithelial cells after a 12 hour fast suggesting an "exit block" similar to the abnormality caused by puromycin administration in rats. Furthermore, we have found that the structurally related antibiotic paromomycin causes xylose and sucrose malabsorption and similar histologic changes as neomycin while tetracycline does not (unpublished data). This suggests that non-absorbable aminoglycoside antibiotics as a class possess the ability to significantly affect mammalian intestinal epithelial cells. The data are consistent with an effect on protein synthesis, however direct proof is lacking.

The exact nature of the "bacteria-like" structures within the macrophages during neomycin administration awaits electron microscopic study. Their appearance and staining characteristics by light microscopy are consistent with bacteria. Although we were unable to culture significant numbers of organisms in intestinal tissue, interpretation is difficult. It is possible that bacteria were dead or in altered form, such as protoplast or L-form and therefore difficult to grow. This has been suggested in Whipple's Disease where bacteria in and around macrophages have been identified morphologically but culture studies have been inconclusive. It is also possible that these structures are merely cellular debris. Whatever their true identify, absorptive abnormalities correlate temporally with epithelial cell damage rather than the appearance and disappearance of these structures.

Summary:

Thirty normal Thai subjects were given oral neomycin and intestinal function and structure studied sequentially. Within 6 hours of a single dose of neomycin, xylose and sucrose were malabsorbed. Intestinal epithelial cells appeared injured and histochemical abnormalities were found. Fat malabsorption occured and an apparent epithelial cell "exit block" was produced. "Bacteria-like" bodies were noted to accumulate within macrophages in the lamina propria. All changes reverted to normal after the drug was stopped. It is suggested that neomycin may directly affect protein synthesis in the human intestinal epithelial cell, and that this may be the underlying pathogenesis of neomycin-induced malabsorption.

Table I

Effect of Chronic Neomycin Administration
On the 25 Gram D.Xylose Tolerance Test

Pt.	Dose of Neomycin (gm per day)	Urine Xylose, gm per 5 hrs			2 Hour Serum Xylose, mg per 100 ml		
		Control	Neomycin	Recovery	Control	Neomycin	Recovery
SC	1	8.5	2.4	8.0			
WI	1	9.6	2.7	7.3			
JA	2	5.4,4.1	1.2	3.7	56.1	[illegible].0	45.L
PB	2	5.2	3.8	7.3			
SO	3.5	4.3, 6.4	0.[illegible],0.5,2.3	3.5,3.2	22.[illegible]	[illegible],8.0,14.3	32.2, 37.7
BO	4	6.8, 4.9	1.7	5.8	60,2,	[illegible]3.4	38.6
CC	4	6.6	2.0,1.4,2.3	4.7,6.2	31	9.6,23.9,14.3	18.4, 47.8
LA	4	9.6 ·	2.5	9.8	47.8	19.5	27.4
MA	4	7.7	3.3	6.2	35.1	14.3	30.6
UR	4.0	4.7,4.3, 4.4,6.0	2.0,2.4	3.7	35.7, 31.2 36.4, 41.0	15.9, 22.9	27.1
AM	4.5	6.3,5.7	1.5,2.3	4.8,4.9 8.6	42.1, 43.6	19,26.1	41.9,45.7,51.8
CH	4.5	7.2,6.9	3.4,4.0	7.0,6.4,6.3	32.4, 40.9	19.8,26.3	34.7,37.2,28.3
SU	4.5	6.4,6.5	2.5,2.6	6.1,5.5,6.0	39.7, 34.5	15.2,18.6	42.4,31.5,31.1
SW	4.5	6.0,5.1	1.8,1.3	4.5,4.6,4.0	46.[illegible], 45.3	23.8,22.7	45.1,45.7,40.2
CL	6	4.0,3.5	1.1	3.[illegible],3.8	40.8, 26.1	14.4	35.4,30.2
LJ	6	4.6,4.6	2.8,1.1	4.2,4.7	39.6, 28.7	16.6,14.4	34.5,34.8
SR	8	4.4,4.3	1.3	2.0,3.9,5.7	56.7, 45.9	16.2	2[illegible].7,34.8 45.7
Mean ± 1 SD		6.26 ± 1.60	2.10 ± 0.75	5.64 ± 1.76	39.60 ± 7.0	17.44 ± 3.83	35.7 ± 5.92

Table II

Acute Effect of a Single 2 Gram Dose Neomycin on the Xylose Tolerance Test

Patient	Urine, gm per 5 hours		2 Hour Serum, mg per 100 ml	
	Control	Neomycin	Control	Neomycin
KA	6.6	4.6	49.6	21.4
KI	5.8, 5.0	3.9	50.5, 53.6	24.5
PA	4.6, 4.9	3.9	43.5, 42.2	43.4
SA	3.1	2.4	36.0	20.9
SR	5.7	0.9	52.7	37.2
WA*	6.2	4.2	—	—
21	5.2	3.1	54.1	28.4
22	6.4	4.1	45.8	28.4
23	5.2	5.0	39.6	25.9
24	6.0	4.7	56.3	40.9
25	5.0	0.3	47.9	25.1
Mean ± 1 S.D.	5.42 ± 0.94	3.37 ± 1.5	47.70 ± 6.35	29.61 ± 7.74

* Intraduodenal administration of 25 gm d-xylose before and 2 hours after intraduodenal administration of 1 gm neomycin.

Table III

Acute Effect of a Single Dose of 2 Grams of Neomycin on the Sucrose Tolerance Test

Patients	Maximum Rise Blood Glucose, mg. per 100 ml			
	Control	Hours Post Neomycin		
		6	18	30
BO	93,64	23	76	91
JA	41,33	6	34	6
KA	65	51		80
KI	53	37	—	35
NI	116	90*	40	—
PA	80	13	—	55
SA	149	17*	59·	—

* 2 hours post neomycin

· 10 hours post neomycin

Table IV

Oral Water Load

Pt.	Control				Neomycin				Recovery			
	Uosm mOsm/L	Cosm ml/min	Max. CH_2O ml/min	Time to Max. Diuresis minutes	Uosm mOsm/L	Cosm ml/min	Max. CH_2O ml/min	Time to Max. Diuresis minutes	Uosm mOsm/L	Cosm ml/min	Max. CH_2O ml/min	Time to Max. Diuresis minutes
SY	50	2.4	11.3	80	54	2.1	8.0	80	59	2.5	9.5	80
MY	60	2.2	7.9	60	64	2.1	6.4	80	59	2.6	9.8	80
PS	70	2.2	6.8	105	74	2.2	6.2	80	65	1.8	6.0	80
NT	51	1.5	7.2	80	50	1.5	6.3	80	63	1.6	5.6	80
SA	66	2.3	6.8	80	81	1.6	3.9	80	85	2.1	5.1	80
TB	48	1.7	8.4	80	54	1.6	6.8	95	62	2.3	8.1	80

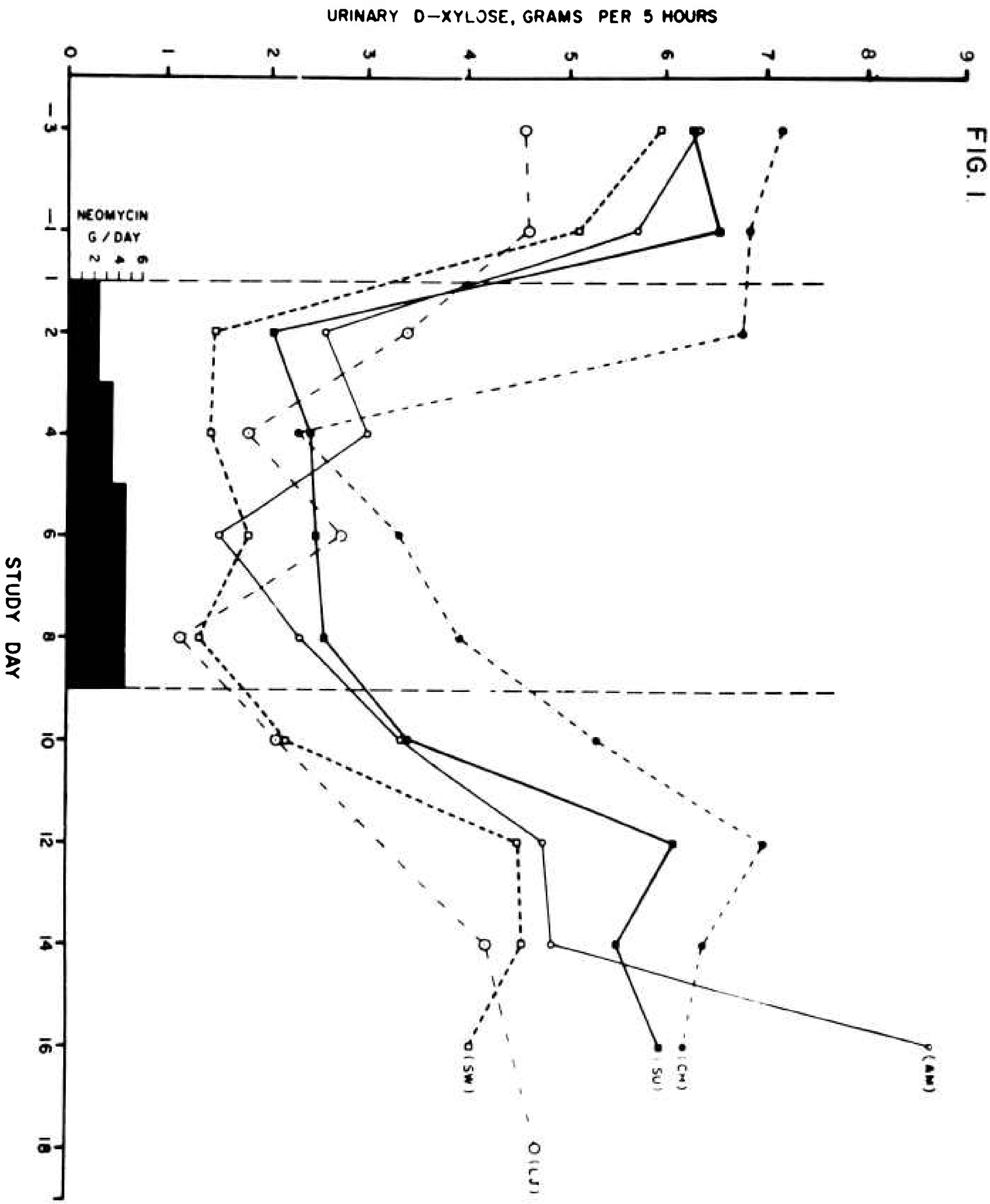
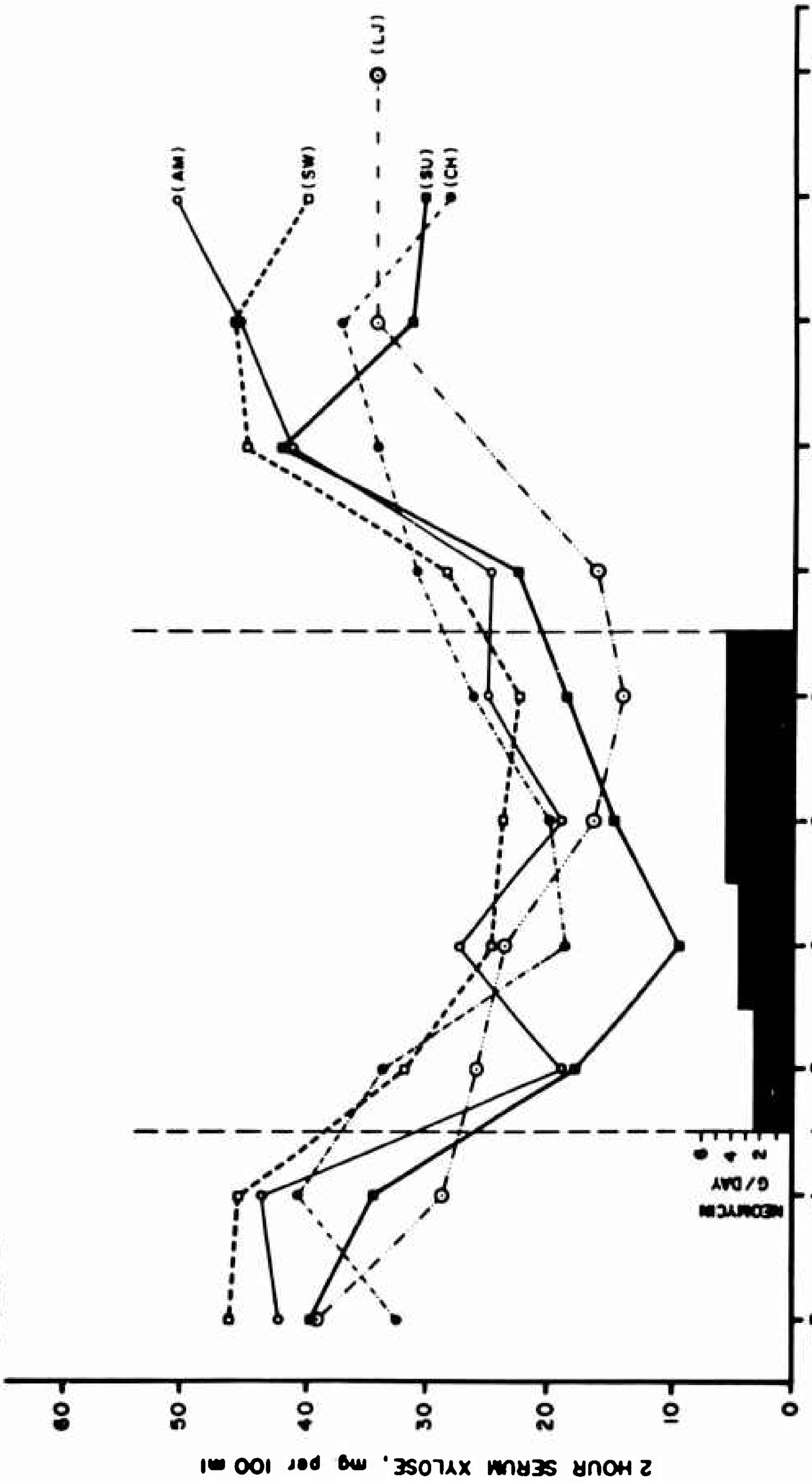

URINARY D-XYLOSE, GRAMS PER 5 HOURS
0
1
2
3
4
5
6
7
8
9
NEOMYCIN
G / DAY
2
4
6
-3
-1
2
4
6
8
10
12
14
16
18
STUDY DAY
(AM)
(CH)
(SU)
(SW)
(LJ)

FIG. I.

FIG. 2.



2 HOUR SERUM XYLOSE, mg per 100 ml
0
10
20
30
40
50
60
STUDY DAY
-3
-1
2
4
6
8
10
12
14
16
18
NEOMYCIN G/DAY
8
4
2
(AM)
(SW)
(SU)
(CH)
(LJ)

FIG. 3. EFFECT OF NEOMYCIN ON SUCROSE ABSORPTION

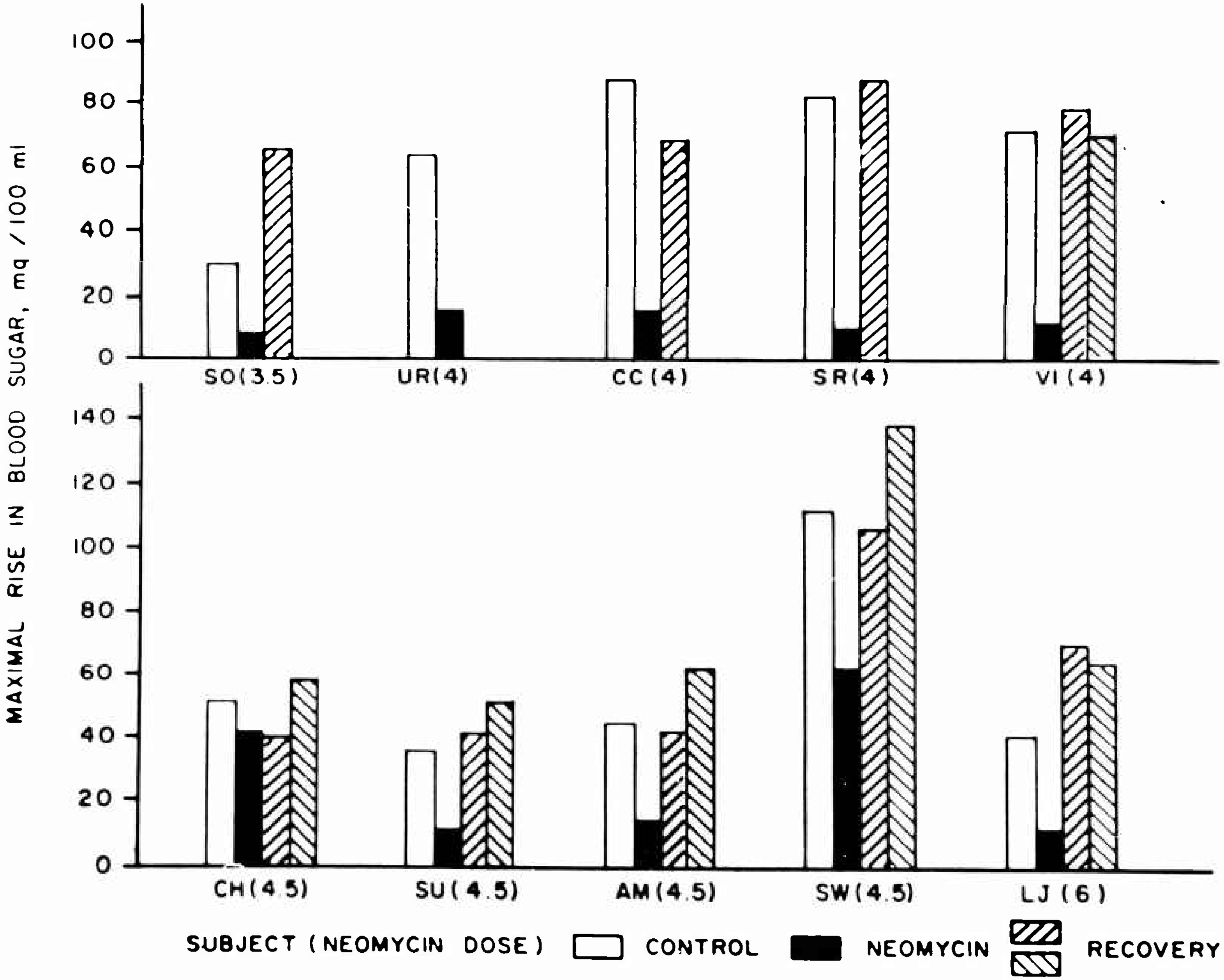
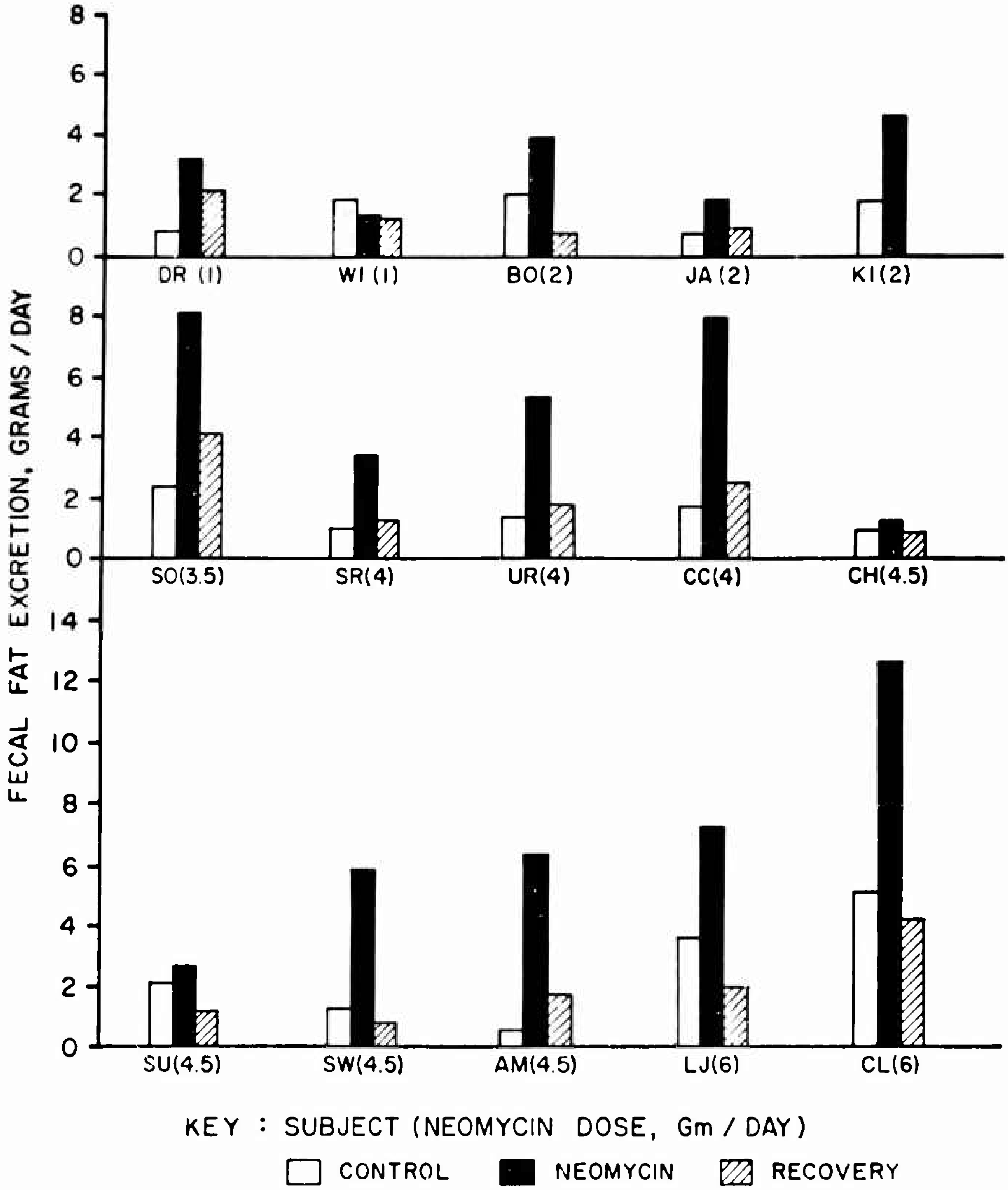


FIG.4. EFFECT OF NEOMYCIN ON FECAL FAT EXCRETION



Title: Malabsorption Due to Paramomycin

Principal Investigator: Gerald T. Keusch, M.D.

Associate Investigators: Richard D. Buchanan, Capt, MC
Frank J. Troncale, Capt, MC

Paramomycin (Humatin[R]) is a non-absorbable antibiotic structurally related to neomycin. The antibacterial spectrum of the two drugs is similar and their clinical applications are generally the same.

Neomycin is known to produce reversible malabsorption in man (1). We have recently shown that malabsorption of xylose and sucrose begins within 6 hours of a single small dose of neomycin in Thai people (2). Histologic and enzyme histochemical abnormalities of the small bowel epithelium occur. Bacteria-like bodies accumulate within macrophages in the lamina propria with continued drug administration. All changes revert to normal when neomycin is stopped.

The present study was undertaken to evaluate the potential of paramomycin to induce functional and structural abnormalities of the small bowel in man. Malabsorption due to paromomycin administration is, to our knowledge, previously unreported.

Material and Methods:

Subjects-Seven normal Thai females, mean age 19.1 (16-29) years, with no history of recent antibiotic ingestion gave informed consent for the studies described below. Subjects were hospitalized and given 2 grams of paromomycin* (approximately 50 mg/kg) daily.

Five hour urinary xylose excretion and 2 hour serum xylose concentration were measured (3) after an oral dose of 25 grams of d-xylose. Sucrose tolerance tests were performed as previously described (2) and the rise in blood total reducing substance (hereafter termed blood sugar) determined by a modification of the Hoffman method adapted to the autoanalyzer (4). Fecal fat content in 4-6 day collections marked by carmine red dye was measured by the method of van de Kamer et al (5). During the study period the usual low fat Thai diet (6) was supplemented with 75 grams of butter daily.

Jejunal biopsies were obtained with the Crosby-Kugler capsule (7) from the region of the ligament of Treitz. Biopsies were taken after a 12-14 hour fast, the morning after a 30-40 gm fat meal. Specimens were oriented on monofilament plastic mesh, placed in Bouin's picroformol acetic fixative and examined with a stereoscopic dissecting microscope. Cryostat sections were stained with Oil-red-O for neutral fat. Paraffin sections were cut at 5 microns and stained with hematoxylin and eosin. Mac Callum-Goodpasture and Feulgen stains were employed on selected material.

* Paromomycin sulfate was a gift of Parke, Davis, and Co.

RESULTS

Absorption Tests:

D-Xylose: Table 1 and figures 1 and 2 show the effect of paramomycin on urinary xylose excretion and serum xylose concentration. No effect was apparent for the first 2 days on drug. Thereafter, there was a significant decrease in both urinary excretion and serum concentration.

Sucrose Tolerance Test: Figure 3 shows the effect of paramomycin on the maximum rise in blood sugar during a sucrose tolerance test. There was considerable variability in the maximum rise during the control period, however, no subject had a flat response (rise in blood sugar of less than 20 mg%). During administration of the drug, 5 of the 7 subjects had a flat response and the maximum rise was blunted in another (PT). No effect occurred in the remaining subject (SW).

Fecal Fat Excretion: There was no effect of the antibiotic on fecal fat excretion (figure 4). In fact, fecal fat content during drug administration was lower than the control value in 4 subjects.

Tissue Examination: Control biopsies conformed to the previously described Thai norm (2). In 6 hour biopsies the epithelial layer contained many polymorphonuclear leucocytes and round cells. There were numerous intracytoplasmic basophilic particles and bacteria-like structures within columnar epithelial cells near the villus tips (figure 5). There was nuclear shrinkage and cytoplasmic clumping, but these changes were quite variable from patient to patient. There were many mitoses in the crypts. The lamina was edematous.

In biopsies obtained on the 7th day of drug administration (figure 6) the villus tips appeared blunt and were crowded with mononuclear phagocytes containing bodies similar to these previously described in neomycin induced malabsorption (2). The alteration in columnar cell morphology described above was present but to a lesser extent.

The columnar epithelial cells appeared normal in biopsies obtained 7 days after the final dose of drug. Phagocytes in the lamina propria were much less conspicuous.

DISCUSSION:

Paromomycin is one of the group of amino-glycoside antibiotics and has structural similarities to neomycin, streptomycin, and kanamycin (9). In its antibacterial spectrum, clincial application, and potential toxicity it most resembles neomycin. It is strange that malabsorption due to paromomycin is not previously reported while several groups of workers have shown the potential of neomycin in inducing malabsorption (1, 10-11). Messinger and Samet (12) did show that paramomycin administration resulted in lowering of serum cholesterol as does neomycin, however, no investigation of intestinal function was conducted,

The present report demonstrates that paramomycin administration causes malabsorption of xylose and sucrose. At the dosage used, no effect on fecal fat excretion was noted. Since diarrhea is not uncommon is subjects receiving more than 3 gm per day of paramomycin (13), it would not be surprising to find steatorrhea with higher doses of drug. This would be consistent with the dose dependent effect of neomycin on fecal fat exeretion (10, 13). Neomycin, however, even in doses of 1-2 grams per day in Thais may cause increased fecal fat excretion (2).

In comparision to our previous data on the effects of neomycin in Thai subjects (2), paramomycin administration causes less disruption of function. Malabsorption within hours of a single dose of paramomycin was not observed. Histologic changes were qualitatively similar but quantitatively less striking than observed with neomycin. Figure 7 shows the effect of the two drugs, administered 4 months apart, in a single patient. It is clear that neomycin produces more profound abnormalities of function.

The mechanism by which neomycin and paramomycin cause malabsorption is not known. The fact that 2 structurally related drugs with the same mechanism of action (15), cause intestinal malfunction suggests that an idiosyncratic reaction is not involved. Suppression of bacterial flora or overgrowth of non-susceptible organisms is unlikely since neomycin induced malabsorption is detectable within hours. Further more, 2.4 grams of tetracycline daily did not produce similar abnormalities of function or structure in Thai subjects (unpublished data). Although other mechanisms, such as alteration of bile salts (16) or abnormal lipolysis (17) have been proposed, it seems probable that these drugs directly affect protein synthesis in human intestinal epithelial cells as they affect sensitive bacterial organisms (2).

The clinical significance of drug-induced malabsorption is uncertain. The relative importance of suppression of nitrogen-forming bacteria and induced azotorrhea by neomycin or paramomycin in the therapy of hepatic coma has not been established. If the latter factor is of importance, neomycin might be the drug of choice since it appears to cause more profound malabsorption. On the other hand, if antibacterial activity is of greater significance, absorption of sugar and fat would probobly be less effected by paramomycin and general nutrition less disturbed. This would be of major importance in poorly nourished cirrhotic patients.

The exact nature and significance of the intercellular bacteria-like bodies which accumulate in macrophages in the lamina propria of the Thai bowel during the administration of either neomycin or paramomycin is unknown. If these are truly bacteria, some breakdown of the normal defenses of the intestinal mucosa would appear to take place. Whether or not this invasion of the lamina propria would increase susceptibility to infection is undetermined. In the short term studies reported here, no serious complications occurred. The effects of long term antibiotic administration, however, in relation to infection and the reversibility of the induced functional defects remain to be studied.

SUMMARY:

The effects of 2 gm per day of paramomycin on intestinal function and structure in 7 normal Thai females was studied. Both xylose and sucrose were malabsorped after 2 days on drug, whereas no effect on fecal fat excretion was found. Histologic abnormalities of the villus epithelial cells, and the appearance of many bacteria-like structures within macrophages in the lamina propria were observed druing drug administration. These functional and structural changes were similar, but less profound than those found in neomycin-induced malabsorption in Thais. All effects were transient and disappeared following cessation of drug administration.

Table I
Effect of Paramomycin on the 25 gram D-Xylose Tolerance Test

Subject	Urine Xylose, grams per 5 Hours			2 Hour Serum Xylose, mg per 100 ml		
	Control	Paramomycin*	Rocovery	Control	Paramymycin*	Recovery
BU	7.05,7.41,5.42	5.21,3.19	1.36	42.9,48,39.4	26.5,23.1	13.1
NO	4.32	1.36	2.48,2.52	37.7	14.3	18.2,25
PN	7.37,6.78,7.78	4.97,5.18	6.76,7.34	56.7,37.8,41.8	31.7,31.7	40.7,51.7
PT	7.35,7.17	4.74	4.64,6.06,4.94	55.4.66	50	50.46.9,49.2
RA	4.63	3.23,3.02	5.91,5.67,6.03	27.7	28.2,26.1	28.3,39.6,40.6
SI	4.80,3.41	3.36,2.47	2.95,4.39	37.3,29.0	22.5,22.5	27.0,33.6
SW	6.94	2.54	4.30	53.6	31	32
Mean	5.88 ± 1.10+	3.42 ± 1.24	4.28 ± 1.52	43 ± 10.8	28.8 ± 3.4	33.1 ± 12.2
"P" value ≠	< 0.01		0.02	< 0.01		NS§

* Day 3-5 on Drug
+ 1 S.D.
≠ Significance of difference between groups compared
§ Not significant at .05 level

FIG. I. EFFECT OF PAROMOMYCIN ON URINARY EXCRETION OF D-XYLOSE, MEAN AND RANGE
25 gram Xylose Test, 5 HOUR URINE COLLECTION

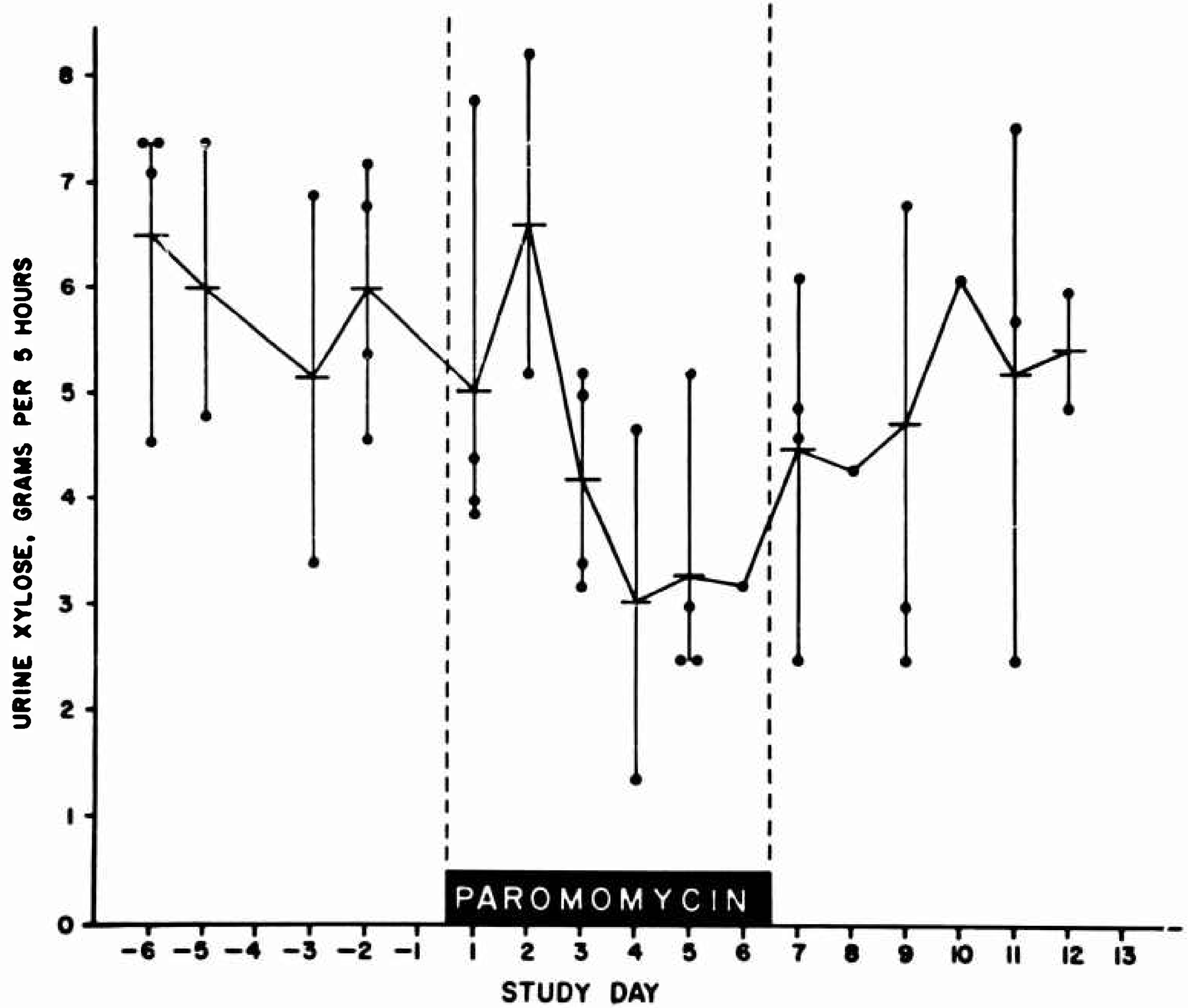


FIG. 2. EFFECT OF PAROMOMYCIN ON SERUM XYLOSE CONCENTRATION 2 HOURS AFTER ORAL ADMINISTRATION OF 25 GRAMS OF D-XYLOSE

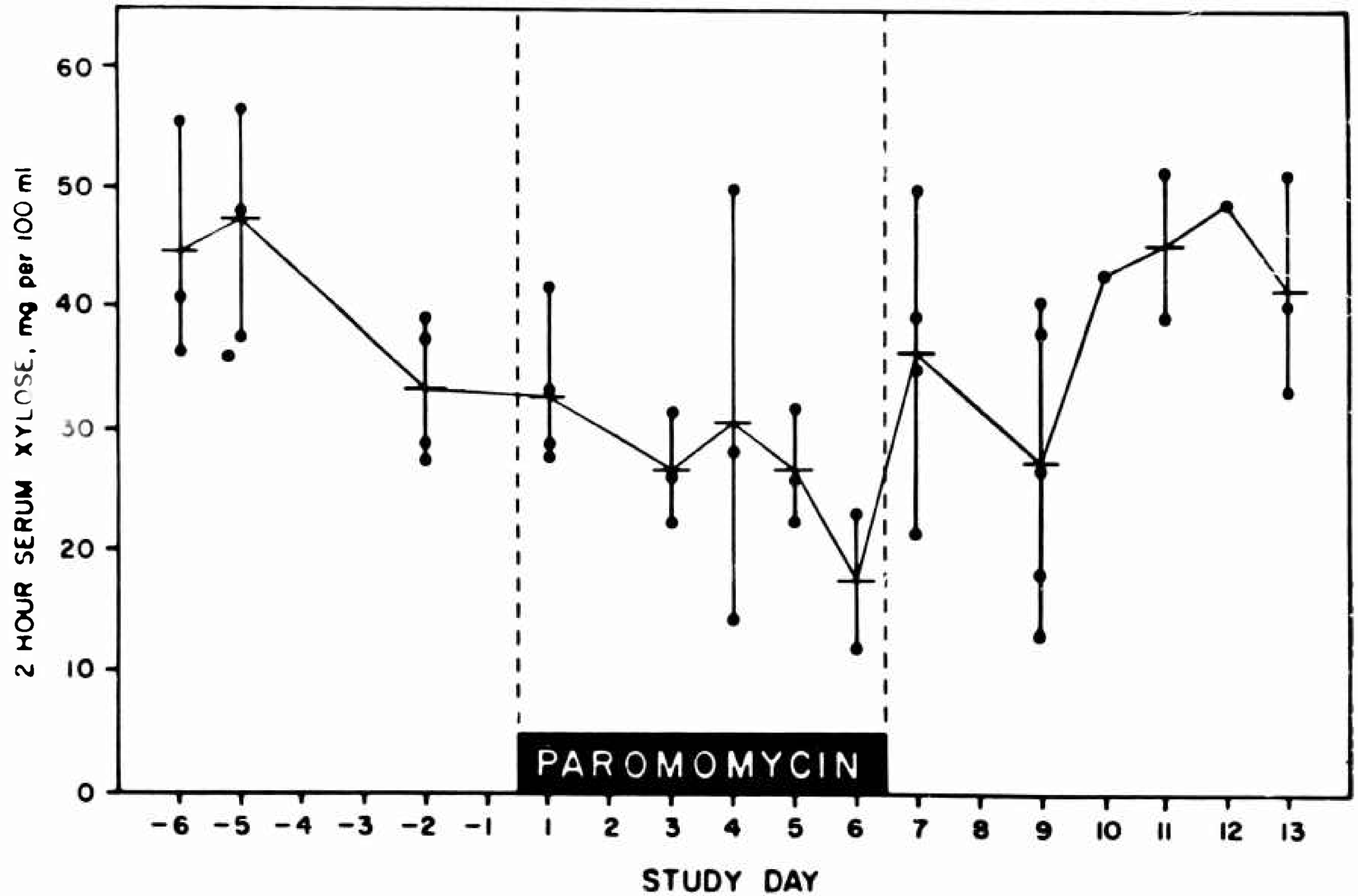


FIG. 3. EFFECT OF PAROMOMYCIN ON THE SUCROSE TOLERANCE TEST. MAXIMUM RISE OF BLOOD SUGAR FOLLOWING SUCROSE, 1.5 gm /KBW, DURING A 90 MINUTE TOLERANCE TEST

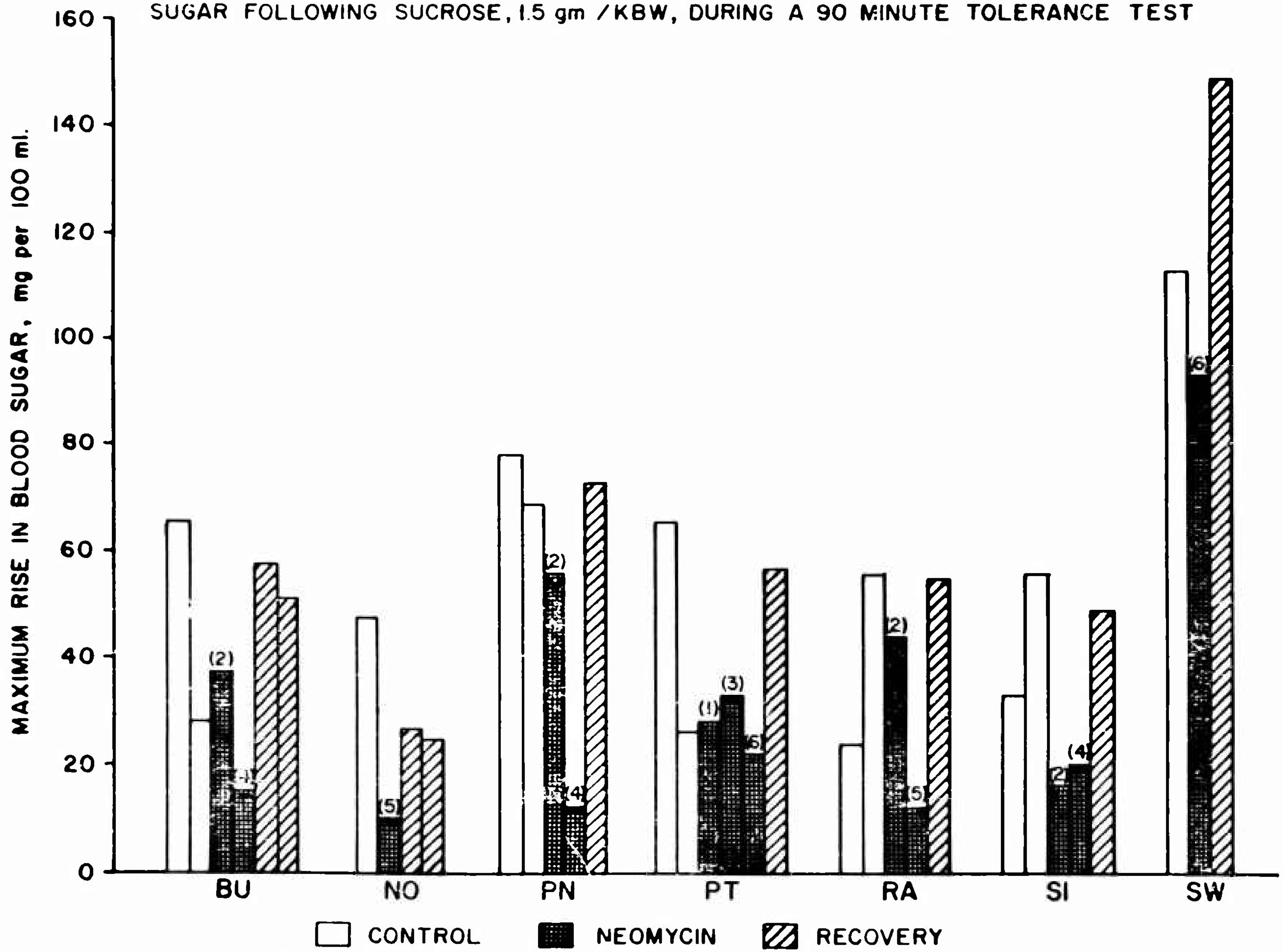


FIGURE IN PARENTHESES INDICATES DAYS ON DRUG WHEN TEST PERFORMED

FIG. 4. EFFECT OF PAROMOMYCIN ON FECAL FAT EXCRETION

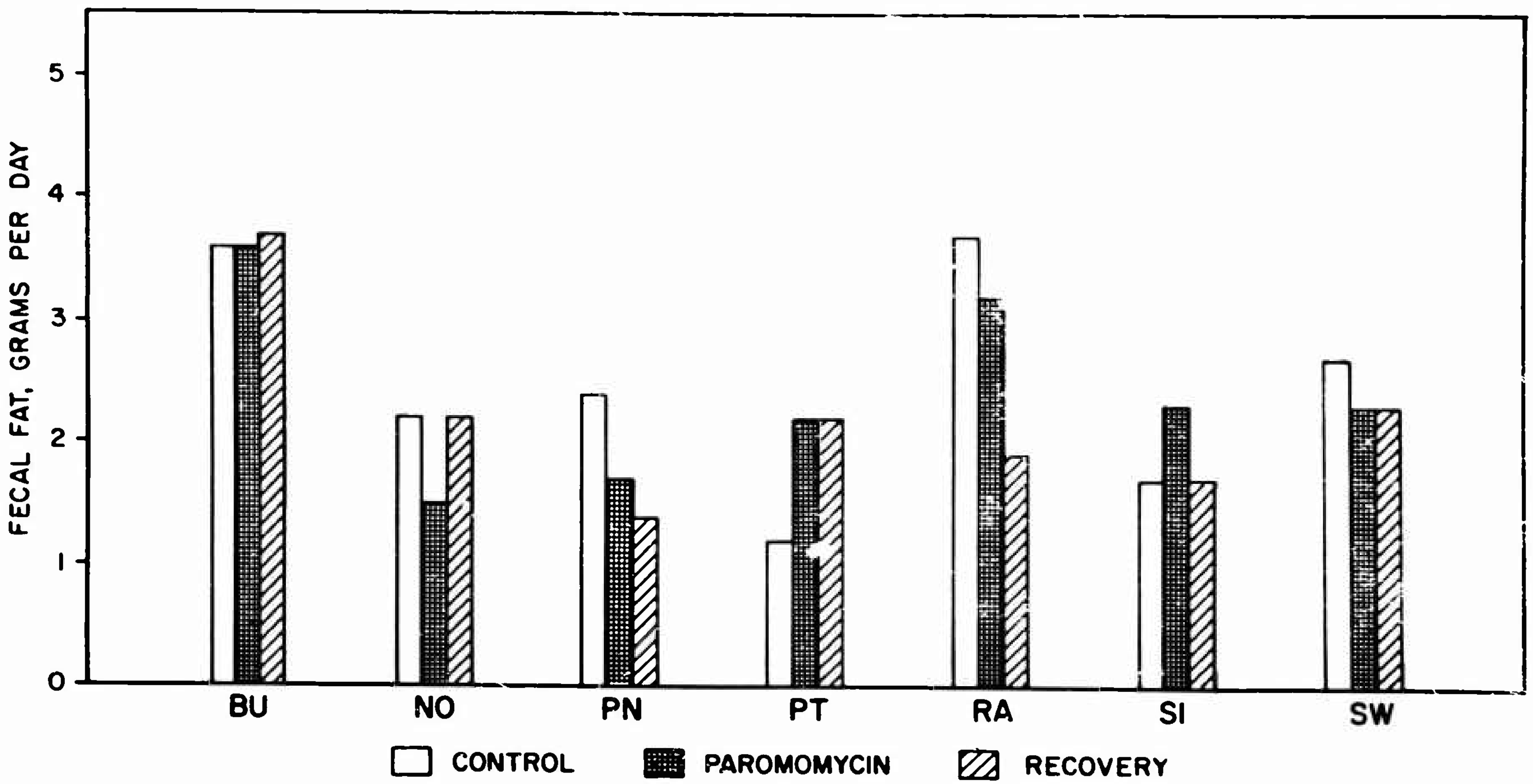


Figure 5. Hematoxylin and eosin, xl, 1000. Control biopsy showing normal jejunal eptithelium on left. Biopsy on right taken 6 hours after oral paromomycin administration. Note disruption of epithelial pattern and cellular debris and intracellular bacteria-like structures.

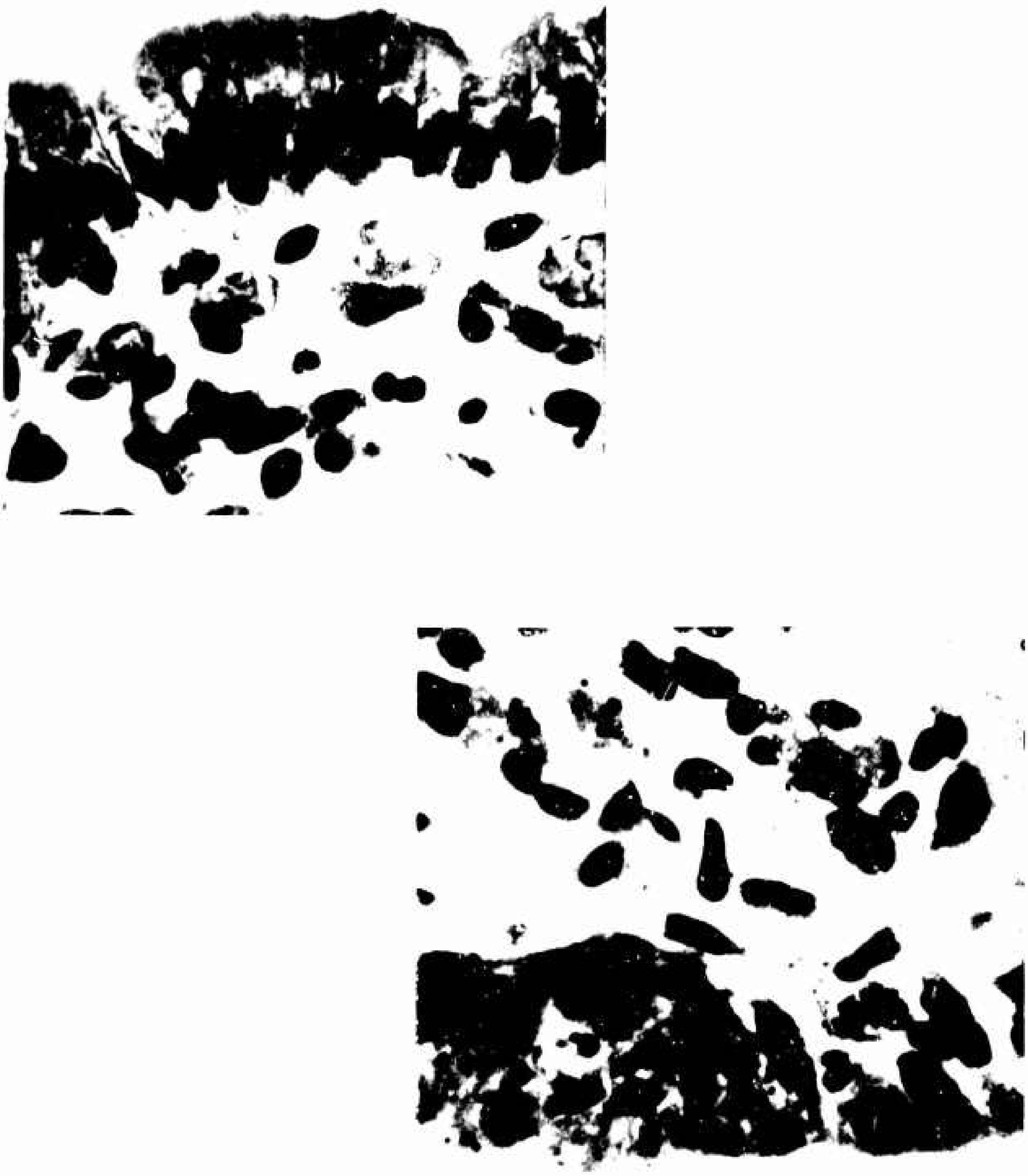
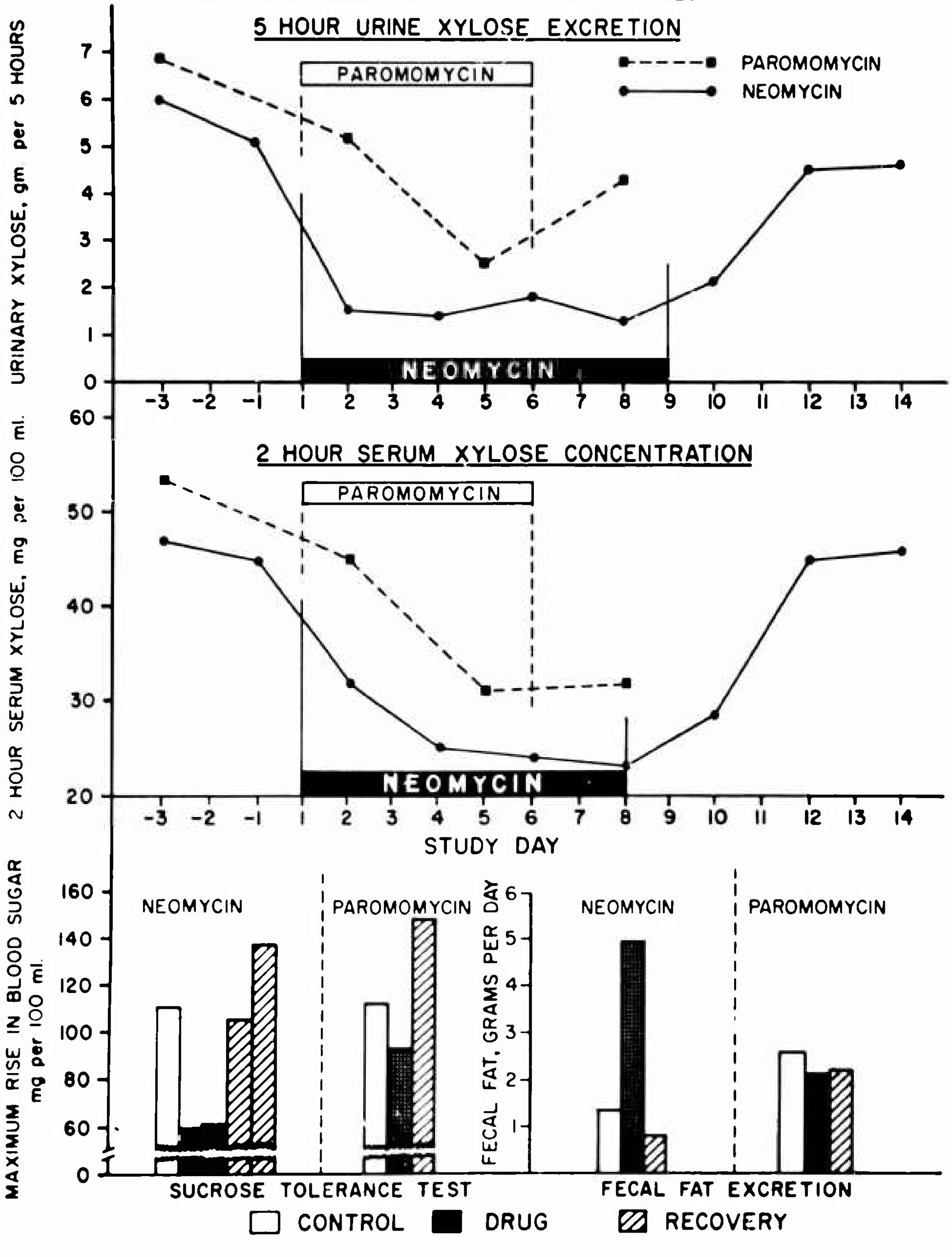


Figure 6. Hematoxylin and eosin, x1,000. Jejunal biopsy taken 7 day after cessation of paramomycin administration. Note return of epithelial pattern toward normal.

FIG. 7. COMPARISON OF THE EFFECT OF NEOMYCIN AND PAROMOMYCIN ON INTESTINAL ABSORPTION IN THE SAME SUBJECT



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1. Title: LACTOSE MALABSORPTION IN THAILAND

I, A Prevalence Study

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Associate Investigator: F. J. Troncale, Capt., M.C.
L. H. Miller, Capt., M.C.
A. G. Plaut, Capt., M.C.
V. Promadhat, M.D.
P. R. Anderson, Ph.D.[1]

Recent studies have shown a high incidence of lactose malabsorption due to lactase deficiency in Bantu tribes in Uganda (Cook and Kajubi 1966), in American Negroes (Bayless and Rosensweig 1966), and in Greek Cypriots living in London (McMichael, Webb and Dawson 1966). These findings have been interpereted to suggest a genetically determined enzyme defect. it is likely that this develops after infancy since congenital lactase deficiency is a serious illness (Holtzel et at 1959). If it is true that lactose malabsorption first occurs after infancy, the differentiation of genetic from environmental factors is critical.

In a previous study of gastrointestinal function in normal adult Thais lactose malabsorption was a universal finding. The present investigation broadens the above observation by studying the prevalence of lactose intolerance, and when possible jejunal lactase activity, in different age groups.

Subjects

Fifty male Thai Marines, 43 pregnant females in all trimesters of pregnancy, and 48 village adults residing near Bangkok comprised the adult group. Most drank no milk at all; a few used a small amount of sweetened condensed milk with coffee. For comparison, data from 58 Peace Corps Volunteers and U.S. Army personnel in Southeast Asia are included. The pediatric group consisted of 85 normal children between 1 and 24 months of age institutionalized at birth because their parents had tuberculosis or Hansen's disease, and 30 children 3-6 years old from a separate orphanage. All drank milk daily, Forty-one unweaned village infants 1-24 months old and 16 non-milk drinking village children, 2½-8 years of age were studied as well.

Methods

Tolerance tersts using lactose or sucrose, 1.5 gm/kg body weight in adults, and 2 gm/kg in the pediatric group, were performed in the fasting state. Glucose tolerance tests utilized half the amount of disaccharide, except for the orphanage group who received 2 gm of glucose/Kg. Venous blood was obtained fasting and at 30, 45, 60, and90 minutes following ingestion of the sugar. In the first 40 adults and 30 children, a 2 and 3 hour blood specimen was also taken. In no case did this change the maximum rise of blood sugar and these samples were thereafter not obtained. Blood total reducing substance, hereafter termed blood sugar, was determined by a ferricyanide method adapted to the autoanalyzer (Hoffman 1937). A rise in blood sugar of 20 mg or more in any of the carbohydrate tolerance tests was considered normal.

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Jejunal biopsy tissue was obtained with the adult (9.5 mm) or pediatric (8 mm) Crosby-Kugler biopsy instrument from the region of the ligament of Treitz after x-ray verification of the position of the capsule. Specimens were wrapped in aluminum foil and immediately frozen and kept at -60°C until determination of disaccharidase activity. Enzyme assay was performed by the method of Dalqvist, modified as previously reported (Sheehy and Anderson 1965). Enzyme activity was calculated as units (u moles of lactose hydrolyzed per minute at 37°) per gram of protein.

Results

Figure 1 shows the maximum rise in blood sugar during the lactose tolerance test in Thai and American adults. The response of the two groups was clearly different. The median rise in blood sugar among the Thais was highest in the Thai Marines. However, only 2 of the 50 marines had a normal test. Table 1 and figure 2 show the results of the lactose tolerance test in institutionalized and village children. The percentage of abnormal responses increased with age. There was a significant decrease in lactose absorption in the institutionalized children after one year of age although milk intake continued. In the village children the change did not occur until after 2 year of age. Lactose absorption in the 1 to 2 year old group was significantly better for the village than the institutionalized children.

Table II show glucose and sucrose and sucrose tolerance test data. There were no abnormal sucrose tolerance tests and in 83 subjects the glucose tolerance test was normal. These results show that sugars other than lactose are normally adsorbed.

Disaccharidase activities are summarized in Table III and Figure 3. In 82% of the 112 Thai specimens examined, lactase activity was less than 5 units per gram of protein. By contrast only 9% of the 23 American specimens were below this level. Maltase and sucrose activity were somewhat lower in the Thai subjects, particularly in the pediatric group. Pediatric biopsies were generally taken proximal to the ligament of Treitz where lower disaccharidase activity is expected (Newcomer, A.D. and McGill D.B. 1966). Enzyme activity was lowest in the orphanage children. In contrast to all other subjects, tissue specimens in this group were frozen and stored on filter paper and some loss of activity might have occurred.

Table III shows the ratios between median dissaccharidase activities. In the Thai subjects only the maltase/sucrase ratio was similar to the American value, while the sucrase/lactase and maltase/lactase ratios were both high due to disproportionate lowering of lactase activity.

Comments

It is apparent that lactase deficiency and an abnormal lactose tolerance test is present in nearly all Thai adults. Similar data in American Negroes have been interpreted to indicate a genetic etiology for isolated lactase deficiency (Bayless and Rosensweig 1966). In support of this hypothesis is the demonstration of a tribal distribution of lactase deficiency in Uganda (Cook and Kajubi 1966). The data from children above the age of 2 years in the present study could be interpreted to favor a similar etiology in the Thai. However, when one studies Thais between 1 month and 2 years of age two facts emerge. Firstly, lactose absorption is normal in infancy, which is presumptive evidence of normal lactase activity (Dunphy et al 1965, Peternel 1965). While these results are inconsistent with the syndrome of congenital lactase deficiency, they do not rule out delayed expression of a genetically controlled trait. Secondly, the delayed age of onset of lactose malabsorption in village children compared to institutionalized children strongly suggests the intervention of environmental influences. Genetic interaction may or may not be present, but cannot be proven from these data.

Overt protein-calorie malnutrition, which may underlie lactase deficiency (Brock, 1966), was not a factor in the institutionalized infants and children who had adequate milk intake. Enzyme adaptation to

substrate withdrawal cannot be considered in this population either. Repeated diarrhea was observed to be exceedingly common in the infants and might be related to deficiency of lactase (Sunshine & Kretchmer 1965) In 3 of 4 infants with lactose malabsorption shortly after acute diarrhea, a normal test was obtained 3-4 weeks later, while in the 4th the rise in blood sugar went from 0 to 13 mg% (unpublished data).

There are 4 possible explanations for persistent hypolactasia and lactose malabsorption in adult Thais: 1) genetically controlled loss of enzyme activity, 2) irreversible intestinal injury acquired in early childhood 3) continued active intestinal injury 4) acquired intestinal injury in childhood with adaptation to the lactose-free Thai diet. There is little pertinent information available in considering these alternatives. Other racial groups or upper class Thais living in Thailand, or young Thais living abroad have not been studied and the question of genetic influences cannot be fully resolved. The present study indicates that with or without genetic interaction, environmental influences can alter the age at which the abnormality develops.

Brock (1966) has raised the interesting possibility that protein malnutrition in infancy may lead to permanent lactase deficiency. Why lactase should fail to return to normal with therapy in kwashiorkor (Cook and Lee 1966) is unknown. It is difficult to explain how a single enzyme system can be permanently damaged in a rapidly regenerating tissue like the small bowel epithelium.

There is no evidence to bear on the 3rd possibility, persistent injury to the intestinal mucosa. Although the intestinal mucosa in adult Thais is not normal by North American standards and thought to represent a pre-sprue lesion (Sprinz et al 1962), function as assessed by a variety of tests is clearly different than in malabsorptive disease (Troncale et al 1967). The epithelial cell brush border, the site of abnormality in primary deficiency of lactase (Crane 1966), is normal as delineated by histochemical techniques (Bhamarapravathi et al 1967). Epidemiologic data are necessary before a continuous environmental factor can be implicated.

The 4th possibility involves two concepts. Several authors have related lactase deficiency to lack of substrate in the diet (Durand 1965, Cuatrecases et al 1965). The Thai adult diet contains little milk or milk products. However, the data presented here indicate that hypolactasia may develop while milk consumption is uninterrupted. It is possible that loss of lactase activity may occur in chilhood due to an unknown cause as discussed above and that low activity is sustained because of secondary adaptation to absence of substrate in the adult diet. The inducibility of lactase in healthy young adult Thais was studied by us and will be reported separately (Keusch et al).

Summary

One hundred forty one normal Thai adults, 172 normal Thai infants and children, and 58 Americans in Southeast Asia were studied to determine the prevalence of lactose malabsorption and lactase deficiency. Lactose malabsorption and lactase deficiency were present in Thai adults. The abnormality was not congenital but occurred after early childhood. Environmental factors are implicated although genetic interaction cannot be excluded.

Table I

Percent of Institution and Village Children with an Abnormal Lactose Tolerance Test*

Age (years)	Percent Abnormal		"p" value† between Institution and Village Children
	Institution Children	Village Children	
(a) 1/12 1	36.8 (49)≠	15 (20)	NS
(b) > 1 2	76.5 (34)	29.6 (21)	0.01
(c) > 2	86.7 (30)	87.5 (15)	NS
"p" a b	< 0.01	NS	
"p" a–c	< 0.01	< 0.01	
"p" b c	NS	< 0.01	

* Maximum rise in blood sugar less than 20 mg%

† Significance of difference between groups compared.

≠ Number of individuals studied in parentheses.

Table II

Mean maximum rise of blood sugar after glucose and sucrose administration in Thais

Group	Glucose			Sucrose		
	No.	Dose	Mean max. rise blood glucose	No.	Dose	Mean max. rise blood glucose
Children						
Orphanage	30	2g/Kg	51.9 ± 20.2 mg%*	10	2g/Kg	51.8 ± 17 mg%
Village	7	1g/Kg	44.9 ± 21.9 mg%*	9	2g/Kg	54.6 ± 18.5mg%
Adults	51	0.75g/Kg	46.0 ± 26.1 mg%*	16	1.5g/Kg	66.9 ± 29.7mg%

* ± 1 S.D.

Table III

Disaccharidase Level*, Median and Range, and Ratios of the Median

Subject Group	Lactase	Maltase	Sucrase	Sucrase / Lactase	Maltase / Lactase	Maltase / Sucrase
Children						
Institution	2.4 (0–47.2)*	170 (43–338)	38 (6–103)	16	50	4.5
Village	2.5 (0–16.26)	265 (83–472)	60 (18.7–140)	24	106	4.4
Thai Marine Adults	2.5 (0.16–58.31)	262 (89–490)	73 (17.8–149)	29	105	3.5
Thai Village Adults	1.7 (0.–7.97)	307 (87–566)	85 (14–142)	50	181	3.6
American Adults	37.5 (2.47–129)	375 (124–600)	108 (34–231)	2.9	10	3.5

* Units per gram of protein (1 unit = 1 umole substrate hydrolyzed/mimute at 37°C)

FIG. 1. MAXIMUM RISE IN BLOOD SUGAR AFTER LACTOSE (1.5 G/Kg) IN HEALTHY ADULTS DURING A 90 MINUTE TOLERANCE TEST

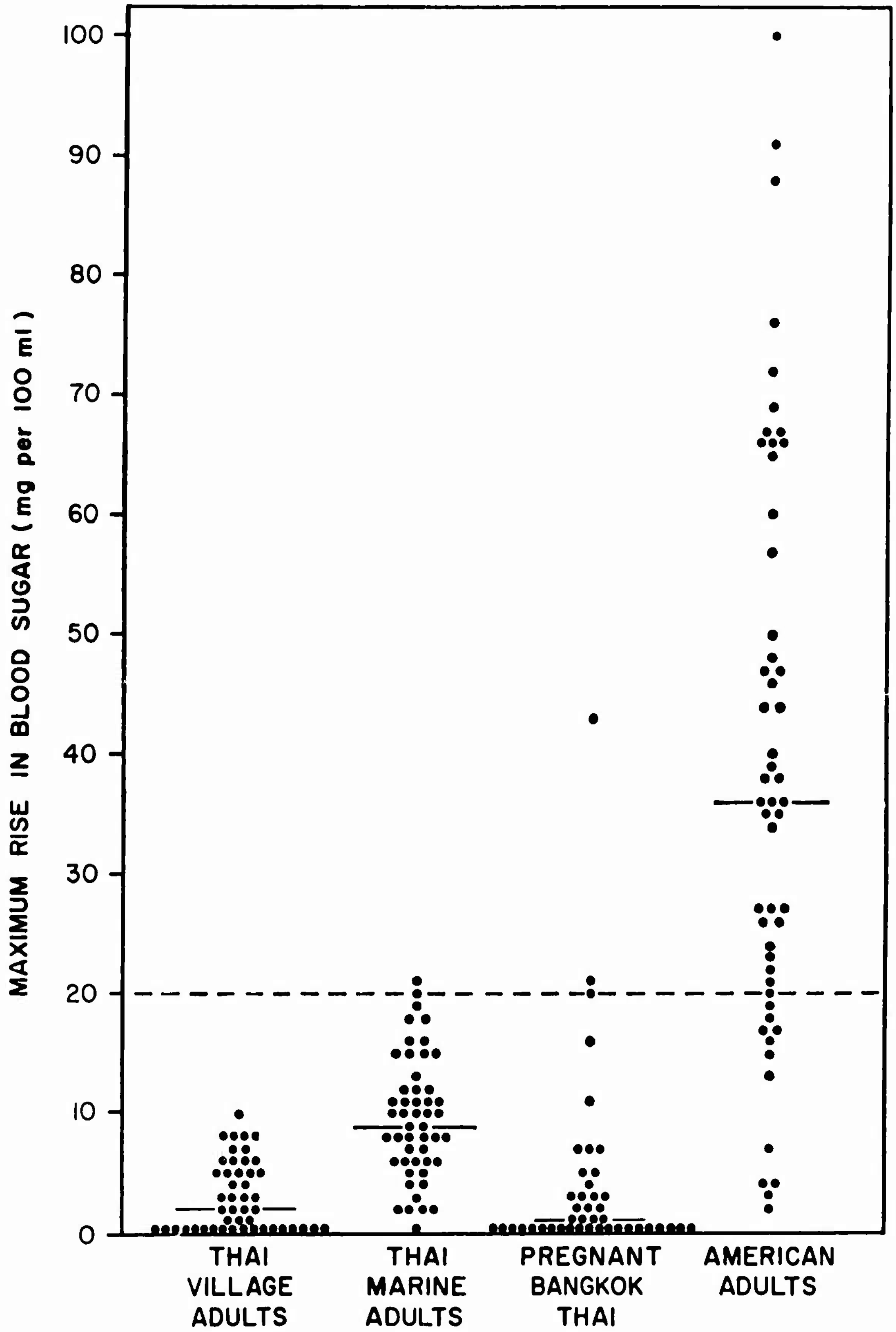
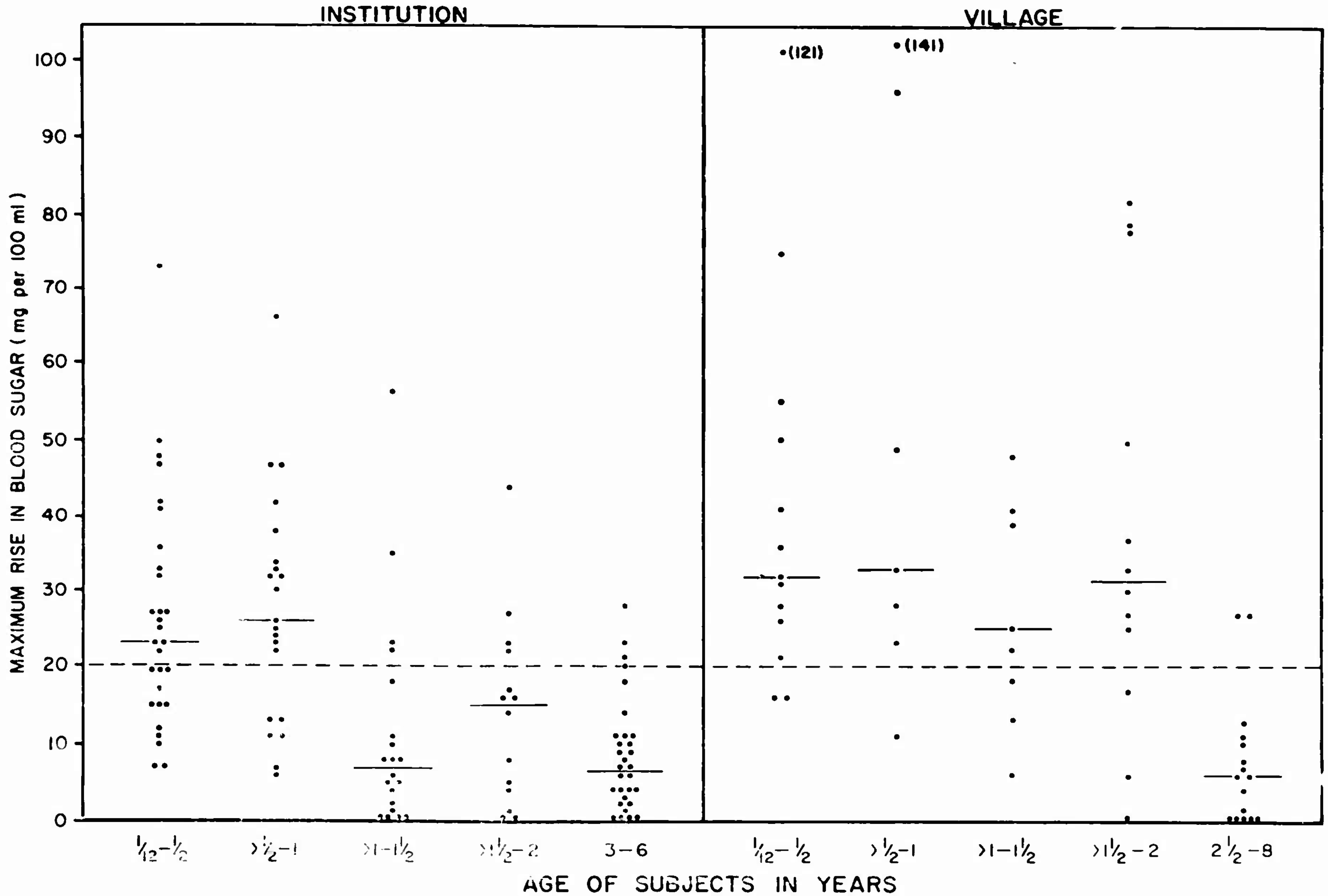


FIG. 2. MAXIMUM RISE IN BLOOD SUGAR AFTER LACTOSE (2 G/Kg) IN INSTITUTIONALIZED AND VILLAGE THAI INFANTS AND CHILDREN DURING A 90 MINUTE TOLERANCE TEST



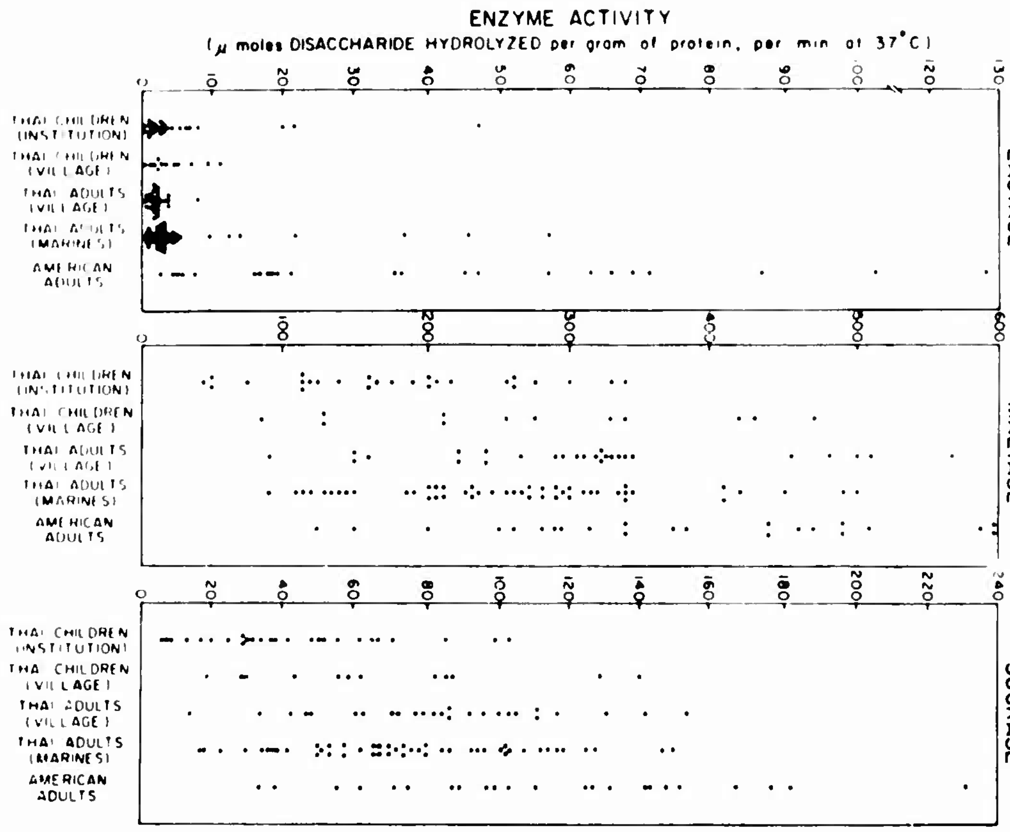


FIG 3 JEJUNAL DISACCHARIDASE ACTIVITY (UNITS PER GRAM OF PROTEIN) IN THAI CHILDREN AND ADULTS AND AMERICAN ADULTS

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I. Title: LACTOSE MALABSORPTION IN THAILAND

II. Enzyme Induction

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Panya Prinyanont, LCDR, RTN[1]

In the previous report we presented evidence that lactose malabsorption and intestinal lactase deficiency occurred after infancy in the Thai population, and was independent of milk drinking. The suggestion has been made that lactase is an adaptive enzyme in man (Cuatrecasas et al 1965). Because we were unable to reject the hypothesis that near universal hypolactasia in adult Thais represented an adaptive phenomenon to the milk-free Thai diet, the present study was undertaken to examine this hypothesis further.

Subject. Fifty male Royal Thai Marine non-commissioned officers and recruits, mean age 22.8 (22-30) years, participated in the study. In 23 subjects there was no history of milk intake while the remaining men took only 1-2 teaspoons of sweetened condensed milk per day in coffee. In no subject was a history of milk intolerance elicited.

Methods: Lactose tolerance tests and disaccharidase assay (Sheehy and Anderson) on jejunal tissue were done. Each subject then began dietary supplementation with 25 grams of lactose twice daily. Complete ingestion of the sugar was verified visually. Lactose feeding was continued for approximately 4 weeks (mean 26.4 days, range 22-38 days) when a repeat tolerance test and biopsy were performed.

Results:

Biopsy. 114 attempts were necessary to obtain 100 tissue specimens for enzyme assay. The reasons for repeat biopsy included failure to pass the pylorus (6), kinking of the tube (2), closure of the capsule without obtaining a specimen (2), and failure of the knife mechanism to close (4). No complications from biopsy were encountered.

The biopsy specimens were studied after fixation in formalin under the dissecting microscope. Special attention was made to discern the presence of various forms of villi i.e., finger, tongue or leaf, short ridge, or convolutions, and the percentage of each form if more than one was present was roughly estimated. Neither long finger villus nor flat mosaic atrophic mucosa was observed in any of the specimens. In forty specimens there was a mixture of leaf and ridge with occasional short fingers (in ten cases). In five cases, the villi consist mostly of convolutions. In another five cases, a mixture of convolution ridge and leaf was noted.

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Clinical Symptoms. Forty six of the 50 subjects had 1-8 watery stools, generally accompanied by cramps as well, within hours of the first lactose tolerance test. In 5 subjects more frequent or softer stools persisted for the entire period of study. Thirty seven subjects experienced symptoms following the second lactose tolerance test.

Tolerance Test. Figure 1 show the maximum rise in blood sugar over fasting in the 50 subjects before and after dietary supplementation with lactose. No significant change occurred after lactose feeding in the group.

Lactase Activity. Figure 2 show lactase activity in units (u moles of lactose hydrolyzed per minute at 37°C) per gram of tissue wet weight and per gram of protein. In 4 specimens (2 pre-and 2 post-lactose feeding) activity of all disaccharidases was extremely low and these results were discarded. There was no significant change in enzyme activity after lactose feeding. Seventy eight of the 96 specimens had low lactase activity, confirming the impression gained from the tolerance test. In 6 subjects normal lactase activity was found in both pre and post lactose specimens associated with abnormal tolerance tests (maximum rise of blood sugar 1-16 mg%). One subject had a normal control lactose tolerance test rise of 20 mg% with low normal lactase activity. The post feeding lactase activity was higher but the rise in the tolerance test was only 10 mg%. Two others had normal lactase before, and 4 others after, lactose feeding with a flat tolerance test (maximum rise of blood sugar of 1-11 mg%).

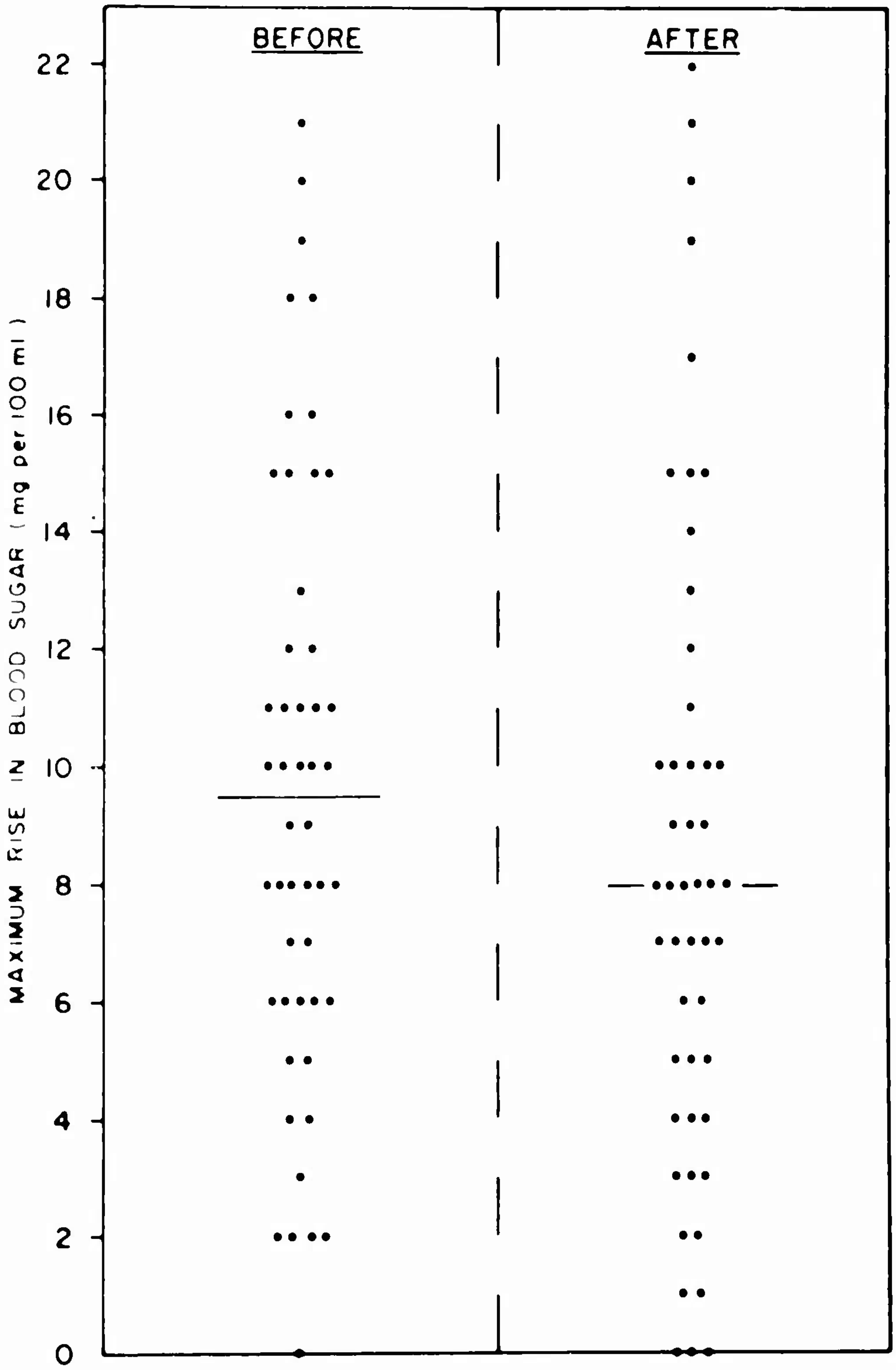
Comments: In several animal species intestinal lactase activity disappears after the weaning period when lactose is no longer a dietary constituent (Holtzel 1965). The California sea lion, whose milk is lactose-free, never develops lactase activity (Sunshine and Kretchmer, 1964). In man the data concerning lactase activity and milk intake are meager. Cuatrecasas (1965) showed an association between low lactase activity, lactose intolerance and the history of no milk ingestion. In addition, when milk was removed from the diet of 2 milk drinkers with normal lactase activity, lactose "absorption" diminished. Prolonged lactose feeding failed to increase absorption, however, in 11 subjects and no increase in enzyme activity occurred in 3 subjects serially biopsied.

The present study, utilizing a sufficiently large population to allow firm conclusions, has failed to show induction of intestinal lactase activity or improvement in lactose absorption in man after prolonged feeding of lactose. It is doubtful that a higher dose of sugar or a longer experiment would have changed the results. Induction of lactase, a very complicated phenomenon, has been extensively studied in *E. coli* (Cohn 1957). Briefly when inducer is introduced into an exponentially growing culture of *E. coli*, synthesis of lactase begins immediately and continues for as long as the inducer remains in the culture medium. When the inducer is removed lactase synthesis stops and enzyme activity per cell decreases as new protein without lactase activity is made. While strict analogy to human intestinal epithelial cells is not possible, the results in the present study give no indication of enzyme induction in man.

Because the intestinal mucosa of Thai subjects looks different than North American subjects (Sprinz et al 1962) the possibility that lactase was not induced in this study because of pre-existing, permanent damage or continuous injury to epithelial cells must be considered (Keusch et al 1967). McMichael et al (1966) have stated that the maltase/lactase ratio in primary (normal histology) lactase deficiency is considerably higher than in secondary (abnormal histology) deficiency because of the marked changes in both enzymes in the latter. The maltase/lactase and sucrase/lactase ratios in our Thai subjects were 5-18 times greater than in the Americans studied by us (Keusch et al 1967) and consistent with a primary type of deficiency. The site of this lesion is, presumably, the brush border of the epithelial cell (Crane 1966). It is not known however, if the nature of the injury in the Thai with non-specific jejunal changes is the same as in the American with normal histology and lactose malabsorption. The poor correlation between lactase deficiency and milk-drinking in these Americans (Littman and Hammond 1965) and the failure to induce enzyme activity in the present study indicate the enzyme adaptation does not occur.

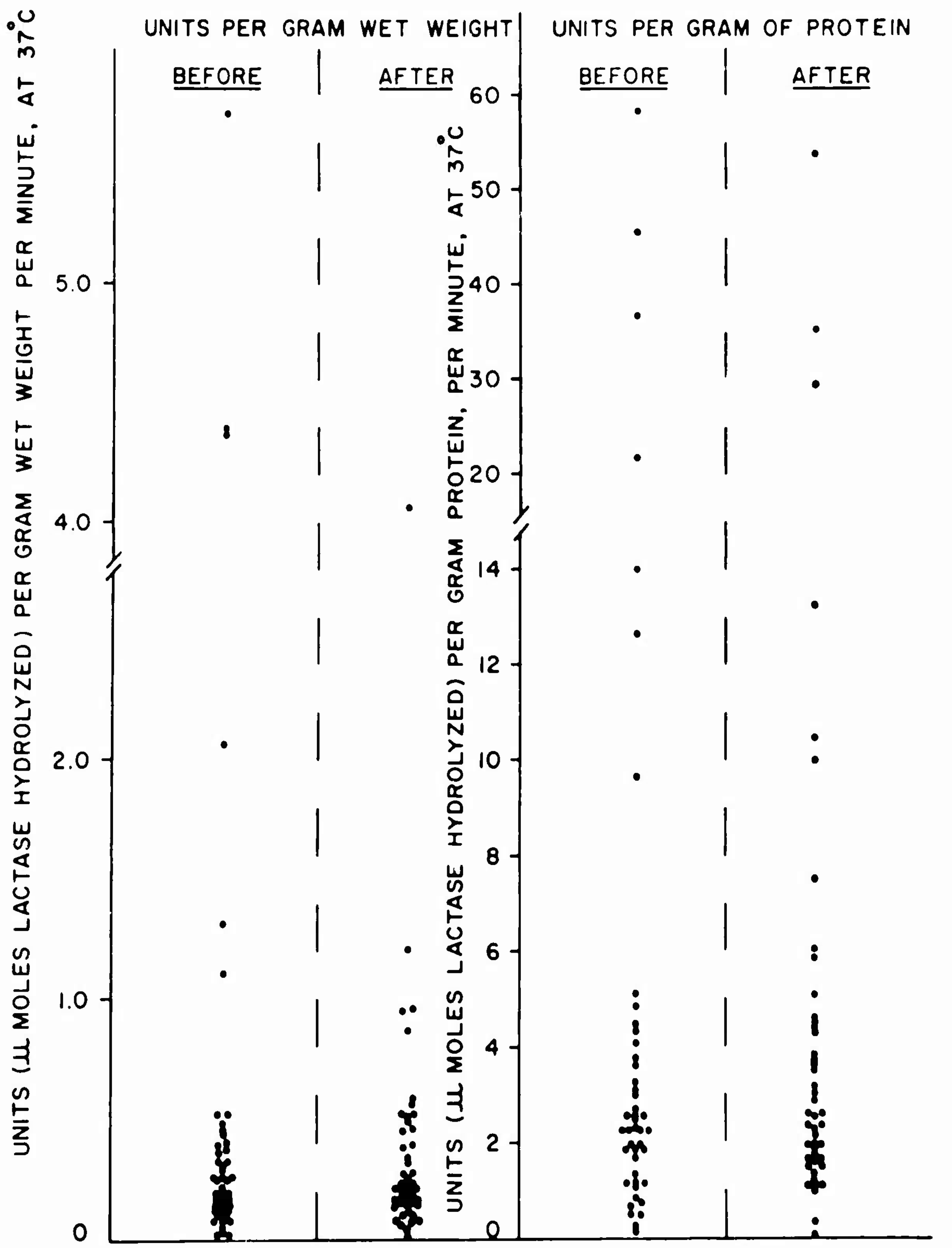
Summary. Induction of intestinal lactase activity was attempted by feeding 50 gm of lactose daily for approximately 4 weeks to 50 male Thai Marines. No change in enzyme activity occurred. The lactose tolerance test was abnormal both before and after the experimental period. It is concluded that lactase is not an adaptive enzyme in man.

FIG. 1. MAXIMUM RISE IN BLOOD SUGAR AFTER LACTOSE (1.5 gm/kg) DURING A 90 MINUTE TOLERANCE TEST BEFORE AND AFTER PROLONGED LACTOSE FEEDING



(HORIZONTAL LINE INDICATES MEDIAN VALUE)

FIG. 2. JEJUNAL LACTASE ACTIVITY BEFORE AND AFTER PROLONGED LACTOSE FEEDING* TO HEALTHY THAI ADULTS



*25 GM OF LACTOSE TWICE DAILY FOR APPROXIMATELY 4 WEEKS

ACKNOWLEDGEMENTS

The assistance of R. Adm. Snit Posakrissana, Surgeon General, RTN, R. Adm. Jinawat Wongpensri, Commanding Officer Sattahip Navy Station, R. Adm. Sophon Suyarnsettakorn, Commandant, RTMC and Capt Samrit Jartinanda, Deputy Director SEATO Medical Research Projects is gratefully acknowledged. Thanks are due LCDR M.C. Pusan Swasdiwatana, MC, RTN, Mrs. Yupa Kasemsant, R.N., Mrs. Pusadee Surisook, R.N., SFC James Turner, U.S.A., Sr. Lt Nuam Pejarat, RTN, and Lt. Saweg Jaigwarng, RTN

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SEATO MEDICAL RESEARCH STUDY ON ECOLOGY OF GIBBONS

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Period of Report:	1 April 1966 31 March 1967

GENERAL INFORMATION

The project on the Ecology of the Gibbon is directed mainly to a consideration of factors important for mainterance and breeding of gibbons in the laboratory and in seminaturalistic reserve areas. The studies comprising the program include observations of animals in natural habitats, experimental ecological studies on a 30-acre island in the Gulf of Siam and an experimental breeding program.

I. NATURAL HABITATS:

The gibbon, Hylobates lar, lives in deciduous forests in northern Thailand. In some sections of the north, restricted forest sites of not more than 10 acres are found with single family groups in them. This is a good deal smaller area than the 50-100 acres believed to be the natural range size of gibbons, and the significance of this observation is that a group can live successfully in a more restricted range than is characteristic in more extended forest areas.

The areas of isolated forest in which gibbons may be found typically border a stream between two hills with vegetation heavy near the water and becoming more sparse toward the top of the hills. Food and water are therefore most plentiful near the stream, and this constellation of factors may tend to keep the animals within the restricted forest areas. It seems likely that given an adequate supply of food, water and cover, gibbons might artificially be kept in rather small forest areas.

These observations were made during a trip to northern Thailand to capture natural groups of gibbons using a drug loaded, gas powered rifle. Gibbons tend not to cross open areas of ground, and it was therefore possible to locate gibbons in the restricted forest areas, drive them to the edge of the forest using a crew of men and shoot at the animals without their leaving the forest.

Of 12 animals seen, it was possible to capture one juvenile by hand and one adult was obtained by proper use of the capture rifle. Six other adults were seen but no shot was possible, and three were killed because of errors in the procedure. Working out the technique will be part of the project during the next year but it seems now as if the following considerations should be part of the method.

Sernylan, an experimental tranquilizer is a useful drug and 1/2 mg. is an adequate dose for an adult gibbon. The effect of the drug is to slow the animal's progress so that he rests in a single tree. He must then be retrieved from the branches. Sernylan is probably superior to drugs which drop the animal

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from a tree since there is a substantial risk to the animal when he falls to the ground. This risk could be reduced some-what by use of a net. However, we found it difficult to use the net accurately in the forest. Sernylan also is not as likely to affect vital functions as some other drugs. Although somewhat slower in action than succinlycholine chloride, the restricted nature of the forest permitted one to follow the animal quite easily in the 5-10 minutes required to bring him to a halt.

In shooting the animal, care must be taken to hit the animal in the abdomen or in the thigh. A 1/2 inch syringe needle is adequate and the needle should have no barb since a barb makes the needle difficult to remove if it becomes embedded in bone. No other type of gun should be used in the procedure, although local hunters may wish to supplement the capture gun with techniques with which they are more familiar.

II. KO KLET KAEO

During the report period, permission was obtained for use of a 30 acre island, Ko Klet Kaeo, as a site for semi naturalistic studies of gibbon ecology. The island is available for ten years under an agreement with the Royal Thai Navy.

The island consists of thickly grown secondary forest throughout, located on steep terrain. Rain is stored in tree holes but otherwise there are no natural water sources and no free water sources are present during the dry season. Despite this fact, there are numerous animals indigenous to the island including at least six species of mosquito (Culex litoralis, Aedes togoi; Aedes qureostrialus; Aedes dissimilis; Aedes (Cancraedes) and Aedes albopictus; two species of rat (Bandicoota indicus; Rattus rattus) and monkeys (Macaca irus).

As an initial effort, eight gibbons were placed on the island. Within one month five had disappeared. The remaining three lived on the island for three months using mostly natural food and water. At the end of this time, two were removed and the remaining animal lived for an additional three months but was found dead soon after the dry season began.

During this initial phase of the project, 20 km. of trails and four small clearings were cut to permit access to all parts of the island. One hundred small feeders and water bowls were distributed widely and a weather station and large trap for monkeys were erected. Two workmen were hired to maintain the feeders and trails and provide security. A new group of gibbons will soon be placed on the island and projects will be initiated concerning the development of territories and ranges.

III. LABORATORY BREEDING PROJECT

Although the technique for maintenmce of gibbons in the laboratory is well worked out, the feasibility of breeding the animal regularly has not been tested. In the natural state, adult gibbon pairs can produce infants at the rate of one every two years, and a few gibbon infants are born in zoos every year.

The laboratory breeding project asks whether, given good laboratory management, gibbons can be bred to produce an adequate number of animals for laboratory investigation. During the report period, twelve 1000 cu. ft. and four 500 cu. ft. outdoor wire mesh cages were constructed at the Phraputabaat facility. Nine compatible pairs of healthy adult gibbons were formed and maintained one pair to a cage. A diet consisting of water and monkey chow is fed ad libitum and is supplemented with oranges, bananas, sweet potatoes, acacia leaves, peanuts, boiled eggs, milk and a vitamin supplement

Under these conditions, seven of the nine pairs have been seen to copulate repeatedly, with copulations observed throughout the day but mainly early in the morning. Not all copulations result in ejaculation. Future work in this project will include observations of conditions under which copulations occur, the prevalence of ejaculation in the male and the development of pregnancies.

SEATO MEDICAL RESEARCH STUDY ON ECTOPARASITES

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Period of Report: 1 April 1966 - 31 March 1967

Objective: To assemble information on the distribution and host-parasite relationships of the ectoparasites of vertebrates in Thailand, especially those of known or suspected importance in transmission of disease: of Public Health importance.

Description: Ectoparasites are removed from mammals and birds collected in connection with various disease studies in Thailand. The ectoparasites are preserved, sorted into major groups and identified at SMRL or submitted to specialists abroad for identification. Aliquots of collections used for inoculations of test animals are given priority in these identifications. Studies on the taxonomy and ecology of the various vertebrate hosts are also conducted.

Progress:

Chiggers: During the period of this report 15,876 microscope slide mounts of chiggers from 633 collections were prepared and identified. Eight species not previously recorded in SMRL collections were found. Fourteen new species of the genus Leptotrombidium are being prepared for publication. A Checklist of the Chiggers (Trombiculidae) of Thailand has been prepared from SMRL and other records, and this host-parasite list is presented below, followed by a map (Figure 1) indicating the known distribution of the vector species of chiggers known to occur in Thailand.

(1) Applied Scientific Research Corporation of Thailand.
(2) Rocky Mountain Laboratory, Hamilton, Montana
(3) Dugway, Utah
(4) Department of Defense, Washington, D.C.
(5), (7), (9) Bishop Museum, Honolulu
(6) Institute for Medical Research, Kuala Lumpur
(8) University of Maryland, Baltimore

CHECKLIST OF THE CHIGGERS (TROMBICULIDAE) OF THAILAND

This list contains only records of Trombiculidae of Thailand reported up to the year 1966.

Family TROMBICULIDAE Ewing, 1944
Subfamily TROMBICULINAE Ewing, 1944
Genus Trombicula Berlese, 1905
Subgenus Trombicula Berlese, 1905

1. Trombicula (Trombicula) calva Domrow, 1962
Localities: Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Nakornsrithamaraj Prov.-Khao Luang Dist. (Ban Na).
Host: Mammal-Hipposideros armiger.

2. Trombicula (Cotrombicula) macclurei Vercammen-Grandjean & Nadchatram, 1963
Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Hosts: Mammals- Mus pahari, Hipposideros sp., Rhinolophus sp..

Subgenus Sasatrombicula Vercammen-Grandjean, 1960

3. Trombicula (Sasatrombicula) keechongi Nadchatram & Mitchell, 1965
Locality: Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Host: Mammal- Rhinolophus sp..

4. Trombicula (Sasatrombicula) siamensis Nadchatram & Mitchell, 1965
Locality: Chiengmai Prov.-Muang Dist. (Doi Suthep).
Host: Mammal- Rhinolophus luctus.

Genus Microtrombicula Ewing, 1950

5. Micromtrombicula munda (Gater, 1932)
Localities: Chantaburi Prov.-Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Muang Dist. (Huai Kaeo), Saraphi Dist. (Ban Khua Moong, Ban San Sai): Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Thong Chai Dist. (K.M. 72.00); Nakornpanom Prov.-Mukdaharn Dist. (Ban Sam Kha); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Nong Bua).
Hosts: Mammals- Menetes berdmorei, Rattus rattus, R. cremoriventer.

6. Microtrombicula spicea (Gater, 1932)
Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham, Muang Ngoi) Hod Dist. (Huai Mae Sanam), Muang Dist. (Ban Chang Khien, Doi Suthep); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.- Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Nakornpanom Prov.-Mukdaharn Dist. (Ban Sam Kha), Tha U-Then Dist. (Ban Pak Thuai); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Nong Bua).
Hosts: Mammals- Tupaia glis, Hylopetes phayrei, Callosciurus erythraeus, Menetes berdmorei, Rattus, rattus, R. rajah.

Genus *Leptotrombidium* Nagayo et al., 1916
Subgenus *Leptotrombidium* Nagayo et al., 1916

7. *Leptotrombidium (Leptotrombidium) akamushi* (Brumpt, 1910)

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Korat Prov..Pak Chong Dist. (Khao Yai National Park); Udornthani Prov..Nong Bua Lam Phu Dist. (Nam Tok Thao To).

Hosts: Mammals-*Tupaia glis*, *Rattus rattus*.

8. *Leptotrombidium (Leptotrombidium) arvina* Schluger et al., 1960

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham); Loei Prov..Wangsapung Dist. (Phu Kra Dung); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Korat Prov..Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00).

Hosts: Mammals-*Tupaia glis*, *Hylomys suillus*, *Crocidura horsfieldi*, *Callosciurus erythraeus*, *Call. finlaysoni*, *Call. caniceps*, *Menetes berdmorei*, *Rattus rattus*, *R. niviventer*, *R. rajah*, *R. berdmorei*; Bird-*Chalcophaps indica*.

9. *Leptotrombidium (Leptotrombidium) binbium* Traub & Lakshana, 1966

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam).

Hosts: Mammals- *Tupaia glis*, *Crocidura horsfieldi*, *Arctogalidia trivirgata*, *Callosciurus erythraeus*, *Call. macclellandi*, *Menetes berdmorei*, *Rattus niviventer*, *Mus pahari*.

10. *Leptotrombidium (Leptotrombidium) deliense* (Walch, 1922)

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Ban Chang Khien, Doi Suthep, Huai Kaeo), San Pa Tong Dist. (Ban Mae Kung Bok, Ban Nong Pung, Ban Tha Lor), Saraphi Dist. (Ban Hua Dong, Ban Khua Moong, Ban Nong Fak, Ban San Sai, Ban San Pa Sak); Chiengrai Prov..Chiengsaen Dist., Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao), Mae Sai Dist. (Ban Doi Tung, Ban San Ton Pui); Cholburi Prov..Sriraja Dist. (Pattaya); Dhonburi Prov..Bangkok Noi Dist. (Bang Plad); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Korat Prov..Muang Dist. (Ban Tha Chang), Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Loei Prov..Wangsapung Dist. (Phu Kra Dung); Lopburi Prov..Muang Dist. (Ban Huai Pong); Nakornsrithamaraj Prov..Chawang Dist. (Ban Tha Phae); Narathivas Prov..Muang Dist. (Ban Thon, Ban Ya Kan), Ra Ngaeh Dist. (Tan Yong Mus); Nong Kai Prov..Muang Dist. (Ban Chom Manee, Ban Non Sang, Ban Tan Chum); Pattani Prov..Na Pra Du Dist. (Nam Tok Huai Sai Kao), Ya-Ring Dist. (Nat Tan Yong, Tan Yong Da Loh); Samuthprakarn Prov..Prapradaeng Dist. (Ban Lad Pho); Saraburi Prov..Prabudhabaht Dist. Nikomsrangtoneng); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Khao Noi, Ban Nong Bua, Ban Nong Si), Nong Bua Lam Phu Dist. (Ban Gud Ling Khor, Nam Tok Thao To); Yala Prov..Muang Dist. (Sam Yaeg A-Sen).

Hosts: Mammals- *Tupaia glis*, *Hylomys suillus*, *Suncus murinus*, *Crocidura horsfieldi*, *Rhinolophus sp.*, *Hipposideros sp*, *Nyctecebus coucang*, *Melogale personata*, *Paradoxurus hermaphroditus*, *Herpestes javanica*, *Tragulus javanicus*, *Hylopestes phayrei*, *Callosciurus erythraeas*, *Call. finlaysoni*, *Call, macclellandi*, *Call. caniceps*, *Menetes berdmorei*, *Rattus rattus*, *R. exulans*, *R. mulleri*, *R. niviventer*, *R. cremoriventer*, *R. rajah*, *R. sabanus*, *R. berdmorei*, *Mus pahari*, *Bandicota indica*; Birds-*Picus canus*, *Picus erythropygius*, *Pollorneum ruficeps*, *Monticola solitarius*, *Geokichla citrina*, *Anthus hodgsoni*.

11. Leptotrombidium (Leptotrombidium) elisbergi Traub & Lakshana, 1966

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam); Ubolrajthani Prov.. Phiboonmangsaharn Dist. (Chong Mek).

Hosts: Mammals-Tupaia glis, Crocidura horsfieldi, Callosciurus erythraeus, Call. caniceps, Menetes berdmorei, Rattus rattus, R. niviventer; Bird-Pellorneum ruficeps.

12. Leptotrombidium (Leptrotrombidium) fulleri (Ewing, 1945)

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), San Pa Thong Dist. (Ban Nong Pung); Loei Prov.. Wangsapung Dist. (Phu Kra Dung); Korat Prov..Pak Chong Dist. (Khao Yai National Park).

Hosts: Mammals- Tupaia glis, Hylomys suillus, Crocidura horsfieldi, Arctogalidia trivirgata, Rattus rattus, R. niviventer, R. rajah.

13. Leptotrombidium (Leptotrombidium) globosa Schluger et al., 1960

Locality: Loei Prov..Wangsapung Dist. (Phu Kra Dung).

Hosts. Mammals- Tupaia glis, Rattus niviventer, Mus sp..

14. Leptotrombidium (Leptotrombidium) hanseni Traub & Lakshana, 1966

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Muang Dist. (Doi Suthep).

Hosts: Mammals- Tupaia glis, Crocidura horsfieldi, Callosciurus macclellandi, Call. caniceps, Mentetes berdmorei, Rattus rattus, R. niviventer, R. rajh.; Bird-Pellorneum ruficeps.

15. Leptotrombidium (Leptotrombidium) micula Traub & Audy, 1954

Locality: Nakornsrithamaraj Prov..Chawang Dist. (Ban Tha Phae).

Host: Mammal- Callosciurus caniceps

16. Leptotrombidium (Leptotrombodium) peniculatum Traub & Lakshana, 1966

Localities: Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Loei Prov..Wangsapung Dist. (Phu Kra Dung).

Hosts: Mammals- Tupaia glis, Melogale personata, Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. sabanus, R. berdmorei.

17. Leptotrombidium (Leptotrombidium) pilosum Traub & Lakshana, 1966

Localities: Chaiyaphum Prov..Muang Dist. (Ban non Khoun, Ban Lat); Chiengmai Prov..Mae Rim Dist. (Ban Don Kaeo).

Hosts: Mammals- Herpestes aurupunctatus siamensis, Rattus sp., Bandicola indica.

18. Leptotrombidium (Leptotrombidium) rapmundi Nadchatram & Upham, 1966.*

Localities: Chantaburi Prov..Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov..Chiengdao Dist. (Ban Tham), Muang Dist. (Doi Suthep); Cholburi Prov..Sriraja Dist. (Bang Phra); Korat Prov..Pak Chong Dist. (Khao Yai National Park); Loei Prov..Wangsapung Dist. (Phu Kra Dung); Nakornnayok Prov..Muang Dist. (Sa Li Ka); Nakornpanom Prov..Tat Panom Dist. (Dong Ma Aek); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov..Muang Dist. (Ban Khao Noi).

Hosts: Mammals- Tupaia glis, Rattus rattus, R. inviventer, R. rajah, R. berdmorei, Bandicota bengalensis, B. indica, Cannomys badius.

19. Leptotrombidium (Leptotrombidium) scanloni Traub & Lakshana, 1966

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Doi Su

* Recorded for first time from Thailand during 1966-67

Theo); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Nakornpanom Prov.-Tha U-Then Dist. (Ban Pak Thuai).
Hosts: Mammals- Tupaia glis, Hylomys suillus, Suncus murinus, Crocidura horsfieldi, Nycticebus coucang, Paradoxurus hermaphroditus, Arctogalidia trivirgata, Callosciurus erythraeus, Call. macclellandi, Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. sabanus, R. berdmorei, Mus pahari; Birds-Picus canus, Picus erythropygius, Pellornaum ruficeps, Anthus hodgsoni.

20. Leptotrombidium (Leptotrombidium) scutellare Nagayo et al., 1921
Localities: Chiengmai Prov.-Hod Dist. (Huai Mae Sanam), Muang Dist. (Doi Suthep), San Pa Thong Dist. (Ban Hua Rin); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao).
Hosts: Mammals- Tupaia glis, Homo sapiens, Melogale personata, Paradoxurus hermaphroditus, Arctogalidia trivirgata, Tragulus javanicus, Callosciurus erythraeus, Call. finlaysoni, Dremomys rufigenys, Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. sabanus, R. berdmorei, Mus sp., Bandicota indica; Birds-Chalcophaps indica, Anthus hodgsoni.

21. Leptotrombidium (Leptotrombidium) striatum Nadchatram & Traub, 1964
Localities: Chantaburi Prov.-Pong Nan Ron Dist. (Klong Ta Kong), Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Doi Suthep); Cholburi Prov.-Sriraja Dist. (Bang Pra Reservior, Pattaya); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.-Tat Panom Dist. (Ban Lao Sam Ran, Dong Ma Aek); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng). Muang Dist. (Phu Khae); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov.-Muang Dist. (Ban Khao Noi, Ban Nong Bua).
Hosts: Mammals-Tupaia glis, Herpester javanica, Callosciurus erythraeus, Call. finlaysoni, Call. caniceps, Menetes berdmorei, Rattus rattus, R. exulans, R. niviventer, R. rajah, R. berdmorei, Bandicota indica; Bird-Pellorneum ruficeps.

Subgenus Lorillatum Nadchatram, 1963

22. Leptotrombidium (Lorillatum) kianjoei Nadchatram & Traub, 1964
Localities: Nakornsrithamaraj Prov.-Chawang Dist. (Nam Tok Tha Phae) Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Udornthani Prov.-Nong Bua Lam Phu Dist. (Nam Tok Thao To).
Hosts: Mammals-Rattus rattus, R. rajah.

23. Leptotrombidium (Lorillatum) mastigophorum Nadchatram 1963
Localities: Chiengmai Prov.-Muang Dist. (Ban Chang Khien, Doi Suthep); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Hosts: Mammals-Rattus rattus, R. niviventer, R. rajah, R. sabanus; Bird-Lonchula punctulata.

24. Leptotrombidium (Lorillatum) oreophilum Nadchatram & Traub, 1964
Localities: Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Hosts: Mammals-Tupaia glis, Rattus rattus, S. rajah.

25. Leptotrombidium (Lorillatum) panitae Nadchatram & Traub, 1964
Localities: Chiengmai Prov.-Muang Dist. (Ban Chang Khien, Doi Suthep).
Hosts: Mammals-Rattus niviventer, R. rajah, Rattus sp.;Bird-Lonchula punctulata

Subgenus Trombiculindus Radford, 1948

26. Leptotrombidium (Trombiculindus) hastata (Gater, 1932)

Localities: Chiengmai Prov..Muang Dist. (Doi Suthep); Korat Prov..Pak Chong Dist. (Khao Yai National Park); Saraburi Prov..Muang Dist. (Phu Khae); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov..Muang Dist. (Ban Chieng pin).

Hosts: Mammals-Tupaia glis, Hylomys suillus, Herpesies javanica, Menetes berdmorei, Rattus rattus, R. exulans, R. niviventer, R. rajah, Bandicota indica.

Genus Chiroptella Vercammen-Grandjean, 1960

Subgenus Chiroptella Vercammen-Grandjean, 1960

27. Chiroptella (Chiroptella) nocticola Nadchatram, 1966

Locality: Chiengmai Prov..Chiengdao Dist. (Ban Tham).

Hosts: Mammals-Hipposideros sp., Myotis adversus. Mus pahari.

28. Chiroptella (Chiroptella) revelae (Audy, 1952)

Locality: Chiengmai Prov..Chiengdao Dist. (Ban Tham)

Hosts: Mammals-Hipposideros sp., Mus pahari.

29. Chiroptella (Chiroptella) sandoshami Nadchatram, 1966*

Locality: Chiengmai Prov..Chiengdao Dist. (Ban Tham).

Host: Mammal-Hipposideros larvatus.

Genus Eutrombicula Ewing, 1938

30. Eutrombicula fieldi Audy, 1956

Locality: Korat Prov..Pak Thong Chai Dist. (K.M. 72.00)

Host: Unknown—Black plate collection.

31. Eutrombicula wichmanni (Oudemans, 1905)

Localities: Chiengmai Prov..Sam Pa Tong Dist. (Ban Tha Lor), Saraphi Dist. (Ban San Sai); Chiengrai Prov..Chiengsaen Dist., Mae Chan Dist. (Mae Chan Mgrket, Ban Kieu Prao); Korat Prov..Pak Chong Dist. (Khao Yai National Park); Nakornsrithamaraj Prov..Chawang Dist. (Ban Tha Phae); Pattani Prov..Na Pra Du Dist. (Nam Tok Huai Sai Kao), Ya Ring Dist. (Nat Tan Yong); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek).

Hosts. Mammals-Tupaia glis, Lepus siamensis, Rattus rattus, R. exulans, R. mulleri, Bandicota indica; Reptiles-Coluber (Natrix) piscator, Veranus sp..

Genus Blankaartia Oudemans, 1911

32. Blankaartia acuscutellaris (Walch, 1922)

Localities: Chiengrai Prov.,Mae Chan Dist. (Ban Kieu Prao); Cholburi Prov:-Sriraja Dist. (Bang Pra Reservior); Lopburi Prov..Muang Dist. (Ban Huai Pong); Narathivas Prov..Muang Dist. (Ban Ya Kan); Yala Prov. Muang Dist..(Ban Lam Mai).

Hosts: Mammals-Rattus rattus, Randicota indica.

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Genus *Neotrombicula* Hirst, 1915

33. *Neotrombicula scorpionis* Lakshana, 1966

Locality: Ublorajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).

Host: "Wood scorpion."

Genus *Siseca* Audy, 1956

34. *Siseca rara* (Walch, 1923)

Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).

Hosts: Mammals-*Tupaia glis, Hylopetes phayrei, Callosciurus erythraeus, Call. finlaysoni, Menetes berdmorei, Rattus rattus*; Bird-*Pitta brachyuma.*

Genus *Myotrombicula* Womersley & Heaslip, 1943

35. *Myotrombicula vercammeni* Nadchatram & Lakshana, 1965

Locality: Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn).

Host: Mammal-*Rattus niviventer?*

Notes: To our knowledge chiggers of the genus *Myotrombicula* have never been recorded from hosts other than bats. So it is suggested that this host record should be accepted with reservations until confirmed by further collections.

Genus *Toritrombicula* Sasa et al., 1953

36. *Toritrombicula hasegawai* (Sasa et al., 1953)

Locality: Korat Prov.-Muang Dist. (Ban Tha Chang).

Host: Bird-*Geokichla citrina*

Genus *Riedlinia* Oudemans, 1914

Subgenus *Riedlinia* Oudemans, 1914

37. *Riedlinia lipoxena* (Womersley, 1952)

Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham); Khon Kaen Prov.-Chumphae Dist, (Pa Dong Larn).

Hosts: Mammals-*Hipposideros armiger, Hipposideros sp.*.

Genus *Trombigastia* Vercammen-Grandjean & Brennan, 1957

Subgenus *Trombigastia* Vercammen-Grandjean & Brennan, 1957

38. *Trombigastia (Trombigastia) harrisoni* (Womersley, 1952)

Locality: Chiengmai Prov.-Chiengdao Dist. (Ban Tham).

Hosts: Mammals-*Mus pahari, Hipposideros sp.*.

39. *Trombigastia (Trombigastia) rousetti* Vercammen-Grandjean & Fain, 1958.*

Locality: Chiengrai Prov.sChiengsean Dist.

Host: Mammal-*Rousettus amplexicaudatus.*

Genus Schoengastia Oudemans, 1910

40. Schoengastia cantonensis Liang et al., 1957
Localities: Nakornpanom Prov.-Tha U-Then Dist. (Ban Pak Thuai); Narathivas Prov.-(Ban Lam Phu, Ban Thon, Ban Ya Kan), Ra Ngaeh Dist. (Tan Yong Mus); Nong Kai Prov.-Muang Dist. (Ban Chom Manee, Ban Non Sang, Ban Tan Chum); Pattani Prov.-Ya-Ring Dist. (Ban Sa Ban); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov.-Muang Dist. (Ban Khao Noi); Yala Prov.-Muang Dist. (Ban Lam Mai).
Hosts: Mammals-Rattus rattus, R. edwardsi, Bandicota indica.

Genus Walchiella Fuller, 1952

41. Walchiella asonluca (Traub & Audy, 1954)
Localities: Chiengmai Prov.-Muang Dist. (Doi Suthep); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Hosts: Mammals-Menetes berdmorei. Rattus rajah.

42. Walchiella hanseni Nadchatram & Lakshana, 1965
Locality: Chiengmai Prov.-Muang Dist. (Doi Suthep).
Host; Rattus sp.

43. Walchiella oudemansi (Walch, 1922)
Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Ubolrajthani Prov.-Phiboonmangsaharm Dist. (Chong Mek); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao).
Hosts: Mammals-Tupaia glis, Hylomys suillus, Menetes berdmorei, Rattus rattus, R. mulleri, R. rajah.

44. Walchiella traubi Womersley, 1952
Localities: Chiengmai Prov.-Muang Dist. (Doi Suthep); Korat Prov.-Pak Chong Dist. (Khao Yai National Park).
Hosts: Mammals-Tupaia glis, Rattus rattus

Genus Aschoschoengastia Ewing, 1946
Subgenus Laurentella Audy, 1956

45: Aschoschoengastia (Laurentella) audyi (Womersley, 1952)
Localities: Chantaburi Prov.-Tha Mai Dist. (wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muan Dist. (Doi Suthep, Huai Haeo), San Kam Paeng Dist. (Ban Sai Mun), San Pa Tong Dist. (Ban Mae Kung, Ban Tha Lor), Saraphi Dist. (Ban Hua Dong, Ban Nong Fak. Ban San Sai); Chiengrai Prov.-Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao, Ban Pha Tang); Cholburi Prov.-Sriraja Dist. (Bang Pra Reservior); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Nakornpanom Prov.-Mukdaharn Dist. (Ban Don, Ban Sam Kha), Tat Panom Dist. (Dong Ma Aek, Nong Yang Sin), Tha U-Then Dist. (Ban Pak Thuai); Narathivas Prov.-Muang Dist. (Ban Ya Kan); Nong Kai Prov.-Muang Dist. (Ban Chom Manee, Ban Nong Sang, Ban Tan Chum); Pattani Prov.-Ya-Ring Dist. (Nat Tan Yong, Ban Sa Ban, Tan Yong Da Loh); Sakolnakorn Prov.-Muang Dist. (Ban Nong Hin); Saraburi Prov.-Prabuddhabaht Dist. (Nikomsrangtoneng); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Khao Noi, Ban Nong Bua, Ban Nong Sai), Nong Bua Lam Phu Dist. (Ban Gud Ling Khor, Nam Tok Thao To); Yala Prov.-Yaha Dist. (Sam Yaeg A-Sen).

* Recorded for first time from Thailand during 1966-67

Hosts: Mammals-Tupaia glis, Hylopetes phayrei, Callosciurus erythraeus, Call. macclellandi, Menetes berdmorei, Rattus rattus (major host), R. exulans, R. niviventer, Rattus sp., Bandicota indica; Bird-Centropus sinensis.

46. Aschoschoengastia (Laurentella) canus Domrow, 1962

Localities: Chiengmai Prov.-Saraphi Dist. (Ban Hua Dong, Ban Khua Moong); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Khao Noi).

Hosts: Mammals-Tupaia glis, Arctogalidia trivirgata, Callosciurus erythraeus, Menetes berdmorei, Rattus rattus.

47. Aschoschoengastia (Laurentella) ctenacarus Domrow, 1962

Locality: Chiengmai Prov.-Chiengdao Dist. (Ban Tham).

Host: Mammal-Arctogalidia trivirgata.

48. Aschoschoengastia (Laurentella) globosa Nadchatram & Domrow, 1964

Locality: Korat Prov.-Pak Chong Dist. (Khao Yai National Park).

Hosts: Mammals-Tupaia glis, Rattus rattus, R. niviventer, R. rajah, Rattus sp.

49. Aschoschoengastia (Laurentella) indica (Hirst, 1915)

Localities: Chantaburi Prov. Pong Nam Ron Dist. (Klong Ta Kong), Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham, Muang Ngoi), Hod Dist. (Ban Bo Luang, Huai Mae Sanam), Muang Dist. (Ban Chang Khien, Doi Suthep Huai Kaeo), San Kam Paeng Dist. (Ban Sai Mun), San Pa Tong Dist. (Ban Mae Kung Bok, Ban Nong Pung, Ban Tha Lor), Saraphi Dist. (Ban Hua Dong, Ban Khua Moong, Ban Nong Fak, Ban Nong Pung, Ban San Kue, Ban San Pa Sak, Ban San Sai); Chiengrai Prov.-Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao, Ban Pha Tang), Mae Sai Dist. (Doi Tung, Ban San Ton Pui); Cholburi Prov.-Sriraja Dist. (Bang Pra Reservior, Pattaya); Dhonburi Prov.-Bangkok Noi Dist. (Bang Plad), Pasicharoen Dist. (Bang Khae); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.-Mukdaharn Dist. (Ban Don, Ban Sam Kha); Tat Panom Dist. (Dong Ma Aek, Nong Yang Sin, Ban Lao Sam Ram), Tha U-Then Dist. (Ban Pak Thuai); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Narathivas Prov.-Muang Dist. Ban Thon, Ban Ya Kan); Nong Kai Prov.-Muang Dist. (Ban Chom Manee, Ban Non Sang, Ban Than Chum); Pathumthani Prov.-Muang Dist. (Ban Chang); Pattani Prov. Muang Dist. (Ban Lam Phu, Pak Nam), Na Pra Du Dist. (Nam Tok Huai Sai Kao), Ya-Ring Dist. (Ban Sa Ban, Nat Tan Yong); Sakolnakorn Prov.-Muang Dist. (Ban Nong Hin); Samuthprakarn Prov.-Prapadaeng Dist. (Ban Lad Pho); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng), Muang Dist. (Phu Khae); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Khao Noi, Ban Nong Bua), Nong Bua Lam Phu Dist. (Ban Gud Ling Khor, Nam Tok Thao To); Yala Prov.-Muang Dist. (Ban Lam Mai), Yaha Dist. (Sam Yaeg A-Sen).

Hosts: Mammals-Tupaia glis, Hipposideros armiger, Arctogalidia trivirgata, Herpestes javanica, Hylopetes phayrei, Callosciurus erythraeus, Call. macclellandi, Call. caniceps, Menetes berdmorei, Rattus rattus, R. exulans, R. niviventer, R. rajah, R. sabanus, R. berdmorei, Rattus sp., Bandicota indica; Bird-Picus canus.

50. Aschoschoengastia (Laurentella) kittii Domrow & Nadchatram, 1964

Localities: Chiengmai Prov.-Muang Dist. (Doi Suthep, Ban Chang Khien).

51. Aschoschoengastia (Laurentella) leechi Domrow, 1962

Localities: Chiengmai Prov.-Hod Dist. (Huai Mae Sanam), Muang Dist. (Doi Suthep); Loei Prov.-Wangsapung Dist. (Phu Kra Dung).

Hosts: Mammals-Tupaia glis, Rattus rattus, R. niviventer, Rattus sp., Mus sp.

52. Aschoschoengastia (Laurentella) lorius (Gunther, 1939)

Localities: Chantaburi Prov.-Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-San Pa Tong Dist. (Ban Tha Lor); Chiengrai Prov.-Mae Chan Dist. (Mae Chan Market); Cholburi Prov.-Sriraja Dist. (Pattaya); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.-Tat Panom Dist. (Dong Ma Aek), Tha U-Then Dist. (Ban Pak Thuai); Narathivas Prov.-Muang Dist. (Ban Thon); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao), Ya-Ring Dist. (Ban Sa Ban, Not Tan Yong).

Hosts: Mammals-Tupaia glis, Menetes berdmorei, Rattus rattus, Rattus sp., Bandicota indica.

53. Aschoschoengastia (Laurentella) roluis (Traub & Audy, 1954)

Localities: Chantaburi Prov.-Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Doi Suthep), San Kam Paeng Dist. (Ban Sai Mun), Saraphi Dist. (Ban Hua Dong, Ban Nong Fak, Ban San Sai); Chiengrai Prov.-Mae Chan Dist. (Mae Chan Market); Cholburi Prov.-Sriraja Dist. (Bang Pra Reservior); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Thong Chai Dist. (K.M. 72.00); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Nakornpanom Prov.-Mukdaharn Dist. (Ban Don), Tat Panom Dist. (Dong Ma Aek, Ban Lao Sam Ran, Nong Yang Sin), Than U-The Dist. (Ban Pak Thuai); Nong Kai Prov.-Muang Dist. (Ban Nong Sang); Sakolnakorn Prov.-Muang Dist. (Ban Nong Hin); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Khao Noi, Ban Nong Bua), Nong Bua Lam Phu Dist. (Nam Tok Thao To, Ban Gud Ling Khor).

Hosts: Mammals-Tupaia glis, Arctogalidia trivirgata, Menetes berdmorei, Rattus rattus, R. exulans, R. cremoriventer, R. rajah, Bandicota indica; Bird-Picus canus.

54. Aschoschoengastia (Laurentella) tafia Nadchatram & Domrow, 1964

Localities: Chantabhri Prov.-Khlung Dist. (Khao Sa Bap), Tha Mai Dist. (Wad Boh Phu): Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Nakornpanom Prov.-Tha U-Then Dist. (Ban Pak Thuai); Narathivas Prov.-Muang Dist. (Ban Thon); Yala Prov.-Yaha Dist. (Sam Yaeg A-Sen).

Hosts: Mammals-Rattus rattus, R. niviventer, R. rajah (major host).

Genus Pseudoschoengastia Lipovsky, 1951

55. Pseudoschoengastia novita Audy & Womersley, 1957

Localities: Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Karat Prov.-Pak Chong Dist. (Khao Yai National Park).

Hosts: Mammals-Tupaia glis, Rattus rajah.

Genus Helenicula Audy, 1954

56. Helenicula kohlsi (Philip & Woodward, 1949)

Localities: Chiengmai Prov.-Hod Dist. (Huai Mae Sanam), Mae Rim Dist. (Ban Don Kaeo), Muang Dist. (Doi Suthep, Huai Kaeo), San Pa Tong Dist. (Ban Tha Lor); Chiengrai Prov.-Chiengsaen Dist., Mae Chan Dist. (Mae Chan Market), Mae Sai Dist.; Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Muang Dist. (Ban Tha Chang); Nakornpanom Prov., Tat Panom Dist. (Ban Lao Sam Ran, Dong Ma Aek, Nong Yang Sin); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Chieng Pin), Nong Bua Lam Phu Dist. (Nam Tok Thao To, Ban Gud Ling Khor).

Hosts: Mammals-Tupaia glis, Callosciurus erythraeus, Menetes berdmorei, Rattus rattus, R. exulans, R. cremoriventer, R. berdmorei, Bandicota indica; Bird-Geokichla citrina.

57. Helenicula lanius (Radford, 1946)

Locality: Chiengmai Prov.-San Sai Dist. (Ban Pong)

Host: Mammal-Bandicota indica.

58. Helenicula mutabilis (Gater, 1932)

Localities: Chiengmai Prov..Muang Dist. (Ban Chang Khien, Doi Suthep, Huai Kaeo), San Pa Tong Dist. (Ban Hua Rin), Saraphi Dist. (Ban San Pa Sak); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Narathivas Prov..Muang Dist. (Ban Lam Phu); Pattani Prov..Na Pra Du Dist. (Nam Tok Huai Sai Kao); Ubolrajthani prov.. Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov..Nong Bua Lam Phu Dist. (Ban Gud Ling Khor); Yala Prov..Muang Dist. (Ban Lam Mai).

Hosts: Mammals-Tupaia glis, Callosciurus caniceps, Dremomys rufigenys, Menetes berdmorei, Rattus rattus, R. rajah, R. sabanus, Bandicota indica.

59. Helenicula scanloni Domrow & Nadchatram, 1964

Localities: Chiengmai Prov..Muang Dist. (Doi Suthep, Huai Kaeo Chiengrai Prov..Mae Chan Dist. (Ban Kieu Prao); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek).

Hosts: Mammals-Tupaia glis, Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. berdmorei

Genus Cheladonta Lipovsky et al., 1955

60. Cheladonta neda Schluger et al., 1960

Localities: Korat Prov..Pak Chong Dist. (Khao Yai National Park); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek).

Hosts: Mammals-Tupaia glis, Menetes berdmorei.

Genus Neoschoengastia Ewing, 1929

61. Neoschoengastia longipes Nadchatram, 1967*

Localities: Nan Prov..Sa Dist. (Ban Pha Hang, Ban Sa Lik).

Hosts: Birds-Anthus hodgsoni, Copsychus malabaricus, C. saularis, Centropus sinensis, Coracias benghalensis, Glancidium cuculoides, Hemipus picatus, Hypothymis azurea, Luscinia cyane, Monticola solitarius, Muscicapa hainana, Phylloscopus fuscatus, Pomatorhinus hypoleucos, Saxicola ferea.

62. Neoschoengastia thomasi (Radford, 1946)

Localities: Chiengmai Prov..Muang Dist. (Doi Suthep); Nan Prov.

Hosts: Birds-Macronus gularis, Copsychus malabaricus.

63. Neoschoengastia struthidia Womersley, 1952

Locality: Nan Prov.

Host: Bird-Pomatorhinus hypoleucos.

Genus Susa Audy & Nadchatram, 1960

64. Susa traubi Nadchatram & Lakshana, 1965

Locality: Chiengmai Prov..Chiengdao Dist. (Ban Tham).

Host: Mammal-Crocidura horsfieldi.

Genus Doloisia Oudemans, 1910

65. Doloisia brachypus Audy & Nadchatram, 1957

Localities: Chantaburi Prov..Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov..Muang Dist. (Doi Suthep); Chiengrai Prov..Mae Chan Dist. (Ban Kieu Prao); Korat Prov..Pak Chong Dist. (Khao Yai National Park);

* Recorded for first time from Thailand during 1966-67

Nakornpanom Prov.-Tat Panom Dist. (Nong Yang Sin), Tha U-Then Dist. (Ban Pak Thuai); Narathivas Prov.-Muang Dist. (Ban Thon); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng); Udornthani Prov.-Muang Dist. (Ban Chieng Pin); Yala Prov.-Yaha Dist. (Sam Yaeg A-Sen).
Hosts: Mammals-Hylomys suillus, Rattus rattus, R. niviventer, R. cremoriventer, R. rajah (major host), R. berdmorei, Bandicota indica.

66. Doloisia browning Audy & Nadchatram, 1957
Localities: Chantaburi Prov.-Khlung Dist. ' ao Sa Bap), Tha Mai Dist. (Wad Boh Phu); Korat Prov.-Pak Chong Dist. (Khao Yai National Park); Narathivas Prov.-Muang Dist. (Ban Thon).
Host: Mammal-Rattus rajah.

67. Doloisia intermedia Audy & Nadchatram, 1957
Localities: Chantaburi Prov.-Khlung Dist. (Khao Sa Bap), Tha Mai Dist. (Wad Boh Phu); Narathivas Prov.-Muang Dist. (Ban Thon); Yala Prov.-Yaha Dist. Sam Yaeg A-Sen).
Host: Mammal-Rattus rajah.

68. Doloisia hooperi Domrow & Nadchatram, 1962
Localities: Chiengmai Prov.-Muang Dist. (Doi Suthep); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek).
Hosts: Mammals-Rattus rattus, R. niviventer, R. rajah, Rattus sp..

69. Doloisia manipurensis (Radford, 1946)
Localities: Chiengrai Prov.-Chiengsaen Dist., Mae Chan Dist. (Ban Kieu Prao).
Hosts: Mammals-Rattus rajah, Cannomys badius.

Genus Schoutedenichia Jadin & Vercammen-Grandjean, 1954

70. Schoutedenichia centralkwangtunga (Mo et al., 1959)
Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), San Kam Paeng Dist. (Ban Sai Mun), San Pa Tong Dist. (Ban Tha Lor), Saraphi Dist. (Ban Hua Dong, Ban Khua Moong, Ban San Sai); Chiengrai Prov. Mae Chan Dist. (Ban Kieu Prao); Cholburi Prov.-Sriraja Dist. (Bang Pra Reservior, Pattaya); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.-Tat Panom Dist. (Dong Ma Aek), Tha U-Then Dist. (Ban Pak Thuai); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng).
Hosts: Mammals,Tupaia glis, Suncus murinus, Crocidura horsfieldi, Herpestes javanica, Callosciurus mocclellandi, Menetes berdmorei, Rattus rattus, R. niviventer, Bandicota indica.

Genus Gahrliepia Oudemans, 1912
Subgenus Gahrliepia Oudemans, 1912

71. Gahrliepia (Gahrliepia) elbeli Traub & Morrow, 1955
Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Mae Rim Dist. (Ban Don Kaeo), Muang Dist. (Ban Chang Khien, Doi Suthep, Huai Kaeo), Saraphi Dist. (Ban Khua Moong, Ban Nam Cho); Chiengrai Prov.-Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao), Mae Sai Dist. (Ban Huai Klai, Ban San Ton Pui); Korat Prov.-Pak Chong Dist. (Khao Yai National Park); Nan Prov.; Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng).
Hosts: Mammals-Tupaia glis, Hylomys suillus, Crocidura horsfieldi, Herpestes javanica, Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. berdmorei, Bandicota bengalensis, B. indica.

72. Gahrliepia (Gahrliepia) fenestrulata Traub & Morrow, 1957

Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Muang Dist. (Doi Suthep); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Kanchanaburi Prov.-Tha Kha Nun Dist. (Bon Hin Laem); Korat Prov.-Pak Thong Chai Dist. (Khao Yai National Park); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Nan Prov..

Hosts: Mammals-Tupaia glis, Menetes sp., Rattus rattus, R. niviventer, R. rajah, Mus sp..

73. Gahrliepia (Gahrliepia) insigne Womersley, 1952*

Locality: Narathivas Prov.-Muang Dist. (Ban Thon).

Host: Mammal: Tupaia glis.

74. Gahrliepia (Gahrliepia) marshi Traub & Morrow, 1957

Localities: Khon Kaen Prov.-Chumphae Dist. (Ban Na Nong Thum); Nakornpanom Prov.-Tha U-Then Dist. (Ban Pak Thuai), Narathivas Prov.-Muang Dist. (Ban Thon); Udornthani Prov.-Muang Dist. (Ban Khao Noi).

Hosts: Mammals-Rattus rattus, R. rajah, R. berdmorei "Wild pig".

75. Gahrliepia (Gahrliepia) mirabilis Schluger et al., 1960

Localities: Chantaburi Prov.-Pong Nam Ron Dist. (Klong Ta Kong), Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Muang Dist. (Huai Kaeo); Chiengrai Prov.-Mae Sai Dist. (Ban San Ton Pui); Cholburi Prov.-Sriraja Dist. (Pattaya); Korat Prov.-Pak Chong Dist. (Khao Yai National Park); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.-Tat Panom Dist. (Nong Yang Sin), Tha U-Then Dist. (Ban Pak Thuai); Saraburi Prov.-Muang Dist. (Phu Khae); Ubolrajthani Prov. Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov.-Muang Dist. (Ban Khao Noi).

Hosts: Mammals-Tupaia glis, Hylomys suillus, Crocidura horsfieldi, Menetes berdmorei, Rattus rattus, R. rajah, R. berdmorei, Bandicota indica, Cannomys badius.

76. Gahrliepia (Gahrliepia) neterella Traub & Morrow, 1955*

Localities: Narathivas Prov.-Muang Dist. (Ban Thon); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao).

Hosts: Mammals- Tupaia glis, Rattus rattus.

77. Gahrliepia (Gahrliepia) tenella Traub & Morrow, 1955

Locality: Chiengrai Prov.-Chaengsaen Dist.

Host: Mammal-Bandicota indica.

78. Gahrliepia (Gahrliegia) tessellata Traub & Morrow, 1955*

Locality: Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao).

Host: Mammal-Rattus rajah.

79. Gahrliepia (Gahrliepia) tylana Traub & Morrow, 1955

Localities: Kanchanaburi Prov., Korat Prov.-Pak Chong Dist. (Khao Yai National Park); Rajburi Prov.-Ban Pong Dist.

Hosts: Mammals-Menetes berdmorei, Bandicota sp. (B. bengalensis?)

Subgenus Walchia Ewing, 1931

80. Gahrliepia (Walchia) alpestris Traub & Evans, 1957

Localities: Chiengmai Prov.-Hod Dist. (Huai Mae Sanam); Chiengrai Prov.-Mae Sai Dist. (Ban Huai Klai).

Host: Mammal-Rattus berdmorei.

*Recorded for first time from Thailand during 1966-67

81. Gahrliepia (Walchia) chinensis Schluger et al., 1960

Localities: Chiengmai Prov..Chiengdao Dist. (Ban Tham), Muang Dist. (Doi Suthep), Saraphi Dist. (Ban Khua Moong, Ban Nong Fak Ban San Fa Sak); Chiengrai Prov.-Chiengsaen Dist., Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao, Ban Pha Tang), Mae Sai Dist. (Doi Tung, Ban Huai Klai); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn). Korat Prov..Pak Chong Dist. (Khao Yai National Park); Loei Prov..Wangsapung Dist. (Phu Kra Dung).

Hosts: Mammals-Tupaia glis, Menetes berdmorei, Rattus rattus, R. exulans, R. niviventer R. rajah, Rattus sp., Mus musculus, Bandicota bengalensis, B. indica.

82. Gahrliepia (Walchia) dismina Schluger et al., 1960

Localities: Chantaburi Prov..Khlung Dist. (Khao Sa Bap), Pong Nam Ron Dist. (Klong Ta Kong), Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov..Muang Dist. (Huai Kaeo), Saraphi Dist. (Ban Nong Fak); Khon Kaen Prov.. Chumphae Dist. (Pa Dong Larn); Korat Prov..Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Loei Prov..Wangsapung Dist. (Phu Kra Dung); Nakornpanom Prov..Tha U-Then Dist. (Ban Pak Thuai); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek); Udornthani Prov..Muang Dist. (Ban Chieng Pin, Ban Nong Bua), Nong Bua Lam Phu Dist. (Bon Gud Ling Khor).

Hosts: Mammals-Tupaia glis, Rattus rattus, R. niviventer, R. rajah, R. sabanus, Rattus sp., Bandicota indica.

83. Gahrliepia (Walchia) disparunguis pingue (Gater, 1932)

Localities: Chiengmai Prov..Muang Dist. (Doi Suthep); Nakornsrithamaraj Prov..Chawang Dist. (Ban Tha Phae); Narathivas Prov..Muang Dist. (Ban Lam Phu, Ban Thon, Ban Ya Kan), Ya-Ring Dist. (Tan Yong Mus); Saraburi Prov..Prabudhabaht Dist. (Nikomsrangtoneng); Pattani Prov..Na Pra Du Dist. (Nam Tok Huai Sai Kao).

Hosts: Mammals-Rattus rattus, R. mulleri, R. niviventer, R. rajah, Rattus sp.

84. Gahrliepia (Walchia) ewingi (Fuller, 1949)

Localities: Chiengmai Prov..Hod Dist. (Huai Mae Sanam); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek).

Hosts: Mammals-Rattus rattus, R. rajah.

85. Gahrliepia (Walchia) ewingi lupella Traub & Evans, 1957

Localities: Chaiyaphum Prov..Muang Dist. (Ban Non Koon); Chantaburi Prov..Pong Nam Ron Dist. (Klong Ta Kong); Chiengmai Prov..Mae Rim Dist. (Ban Don Kaeo), Muang Dist. (Doi Suthep), San Kam Paeng Dist. (Ban Sai Mun), San Pa Tong Dist. (Ban Mae Kung Bok, Ban Tha Lor); Chiengrai Prov..Chiengsaen Dist., Mae Chan Dist. (Mae Chan Market), Mae Sai Dist. (Ban Huai Klai, Doi Tung); Cholburi Prov..Sriraja Dist. (Bang Pra Reservior, Pattaya); Khon Kaen Prov..Chumphae Dist. (Pa Dong Larn); Korat Prov..Pak Chong Dist. (Khao Yai National Park); Lopburi Prov..Muang Dist. (Ban Huai Pong); Nakornpanom Prov..Tat Panom Dist. (Ban Lao Sam Ran, Dong Ma Aek, Nong Yang Sin), Tha U-Then Dist. (Ban Pak Thuai); Nong Kai Prov..Muang Dist. (Ban Chom Manee, Ban Non Sang, Ban Tan Chum); Sakolnakorn Prov..Muang Dist. (Ban Nong Hin); Saraburi Prov..Muang Dist. (Phu Khae); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov..Muang Dist. (Ban Chieng Pin, Ban Dong, Ban Khao Noi, Ban Nong Bua).

Hosts: Mammals- Tupaia glis, Hylomys suillus Herpestes javanica, Herpestes sp., Menetes berdmorei, Rattus rattus, R. niviventer, R. rajah, R. sabanus, R. berdmorei, Rattus sp.- Bandicota bengalensis, B. indica.

86. Gahrliepia (Walchia) isonychia Nadchatram & Traub, 1964

Localities: Korat Prov..Pak Chong Dist. (Khao Yai National Park); Nakornpanom Prov..Tat Panom Dist. (Nong Yang Sin), Tha U-Then Dist. (Ban Pak Thuai); Nong Kai Prov..Muang Dist. (Ban Than Chum); Ubolrajthani Prov..Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov..Muang Dist. (Ban Chieng Pin, Ban Khao Noi, Ban Nong Bua).

Hosts: Mammals-Tupaia glis, Herpestes javanica, Rattus rattus, R. exulans, R. rajah, R. rajah, R. berdmorei, Bandicota indica.

87. Gahrliepia (Walchia) kritochaeta Traub & Evans, 1957

Localities: Chantaburi Prov..Khlung Dist. (Khao Sa Bap), Tha Mai Dist. (Wad Boh Phu); Chiengrai Prov.-Chiengsaen Dist. (Ban Pa Sak Noi), Mae Sai Dist. (Doi Tung, Ban Huai Klai); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00), Si Kiu Dist.; Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Lopburi Prov.-Muang Dist. (Ban Huia Pong); Nakornpanom Prov.-Tha U-Then Dist. (Ban Pak Thuai); Nong Kai Prov.-Muang Dist. (Ban Than Chum); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng); Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium); Udornthani Prov.-Muang Dist. (Ban Khao Noi).

Hosts: Mammals-Tupaia glis, Menetes berdmorei, Rattus rattus, R. exulans, R. niviventer, R. cremoriventer, R. rajah, R. sabanus, R. berdmorei, Rattus sp., Bandicota bengalensis, B. indica.

88. Gahrliepia (Walchia) lewthwaitei (Gater, 1932)*

Localities: Narathivas Prov.-Muang Dist. (Ban Thon, Ban Ya Kan); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao); Yala Prov.-Yaha Dist. (Sam Yaeg A-Sen).

Hosts: Mammals-Rattus rattus, R. rajah (major host).

89. Gahrliepia (Walchia) micropelta Traub & Evans, 1957

Localities: Chaiyaphum Prov.-Muang Dist. (Ban Lat); Chantaburi Prov.-Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Mae Rim Dist. (Ban Don Kaeo), San Pa Tong Dist. (Ban Tha Lor); Chiengrai Prov.-Chiengsaen Dist. (Ban Pa Sak Noi), Mae Chan Dist. (Mae Chan Market, Ban Kieu Prao), Mae Sai Dist. (Doi Tung, Ban Huai Klai, Ban San Ton Pui), Muang Dist. (Ban Pha Tang); Khon Kaen Prov.-Chumphae Dist. (Pa Dong Larn); Korat Prov.-Pak Chong Dist. (Khao Yai National Park), Pak Thong Chai Dist. (K.M. 72.00); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Lopburi Prov.-Muang Dist. (Ban Huai Pong); Nakornpanom Prov.- Tha U-Then Dist. (Ban Pak Thuai); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Sakolnakorn Prov.-Muang Dist. Dist. (Ban Nong Hin); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng), Ubolrajthani Prov.-Phiboonmangsaharn Dist. (Chong Mek, Khong Jium) Udornthani Prov.-Nong Bua Lam Phu Dist. (Ban Gud Ling Khor, Nam Tok Thao To), Muang Dist. (Ban Chieng Pin, Ban Nong Bau).

Hosts: Mammals-Tupaia glis, Rattus rattus, R. exulans, R. nivinventer, R. rajah, R. sabanus, R. berdmorei, Rattus sp., Mus sp., Bandicota bengalensis, B. indica.

90. Gahrliepia (Walchia) rustica (Gater, 1932)

Localities: Chantaburi Prov.-Tha Mai Dist. (Wad Boh Phu); Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Ban Chieng Pin, Doi Suthep, Huai Kaeo), San Kam Paeng Dist. (Ban Sai Mun), San Pa Tong Dist. (Ban Tha Lor), Saraphi Dist. (Ban Don Kaeo, Ban Hua Dong, Ban Khua Moong, Ban San Sai); Chiengrai Proav.-Chiengsaen Dist. (Ban Pa Sak Noi), Mae Chan Dist. (Mae chan Market), Mae Sai Dist. (Doi Tnng, Huai Klai, Ban San Ton Pui); Korat Prov.-Pak Chong Dist. (Khao Yai National Park); Loei Prov.-Wangsapung Dist. (Phu Kra Dung); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae); Saraburi Prov.-Prabudhabaht Dist. (Nikomsrangtoneng); Udornthani Prov.-Nong Bua Lam Phu Dist. (Ban Gud Ling Khor, Nam Tok Thao To); Pattani Prov.-Na Pra Du Dist. (Nam Tok Huai Sai Kao).

Hosts: Mammals-Tupaia glis, Suncus murinus, Crocidura horsfieldi Herpestes javanica, Callosciurus erythraeus, Call. macclellandi, Call. coniceps, Menetes berdmorei, Rattus rattus, R. mulleri, R. niviventer, R. rajah, R. berdmorei, Rattus sp., Mus pahari, Mus sp., Bandicota indica.

Subgenus Schoengastiella Hirst, 1915

91. Gharliepia (Schoengastiella) ligular (Radford, 1946)

Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Muang Dist. (Doi Suthep, Huai Kaeo), San Pa Tong

*Recorded for first time from Thailand during 1966-67

Dist. (Ban Nong Pung); Chiengrai Prov..Mae Chan Dist. (Mae Chan Market, Ban Pha Tang); Cholburi Prov.. Sriraja Dist. (Bang Pra Reservior); Korat Prov..Pak chong Dist. (Khao Yai National Park); Nakornsrithamaraj Prov.-Chawang Dist. (Ban Tha Phae).

Hosts: Mammals-Rattus rattus, R. exulans, R. rajah.

Subfamily LEEUWENHOEKIINAE Womersley, 1944

Genus Odontacarus Ewing, 1929

92. Odontacarus audyi (Radford, 1946)

Localities: Chiengmai Prov.-Chiengdao Dist. (Ban Tham), Hod Dist. (Huai Mae Sanam), Muang Dist. (Ban Bo Kaeo); Nan Prov.-Sa Dist. (Ban Pa Chom Poo, Ban Pha Hang).

Hosts: Birds-Anthus hodgsoni, Centropus sinensis, Copsychus malabaricus, Garrulax moniliger, Lanius collurioides, Luscinia cyane, Monticola solitarius, Muscicapa banyumus, Otus bakkamoena, Pellorneum ruficeps, Pomatorhinus hypoleucos, Saxicola ferrea.

Genus Whartonia Ewing, 1944

93. Whartonia prima Schluger et al., 1959

Locality: Chantaburi Province.

Host: Mammal-Hipposideros armiger debilis.

Mesostigmatid mites:

Approximately 7000 slides of mites other than chiggers were examined during this period. These represented 959 collections, 767 of which were collected by SMRL while the others were made in Thailand by Dr. Elbel's team. The entire lot of material examined were collected from the following provinces: 1) Chantaburi, 2) Chiengmai, 3) Chiengrai, 4) Cholburi, 5) Dhonburi, 6) Khon Kaen, 7) Krabi,8) Loei 9) Lopburi, 10) Nakornrajsrima, 11) Nakornsrithamaraj, 12) Patalung, 13) Phang Nga, 14) Phuket, 15) Ranong, 16) Samuthprakarn, 17) Satool, 18) Saraburi, 19) Songkhla, 20) Trang, 21) Ubolrajthani and 22) Udornthani.

Twenty four families of mites were represented in these collections, but only the slides of mites belonging to six families of wide distribution in Thailand were identified to species. The total number of species of mesostigmatid mites known from Thailand thus far is 48,

List of Mesostigmatid Mites recorded from Thailand

I. Family DERMANYSSIDAE Kolennati, 1859

Dermanyssus gallinae (DeGeer, 1778)

Echinonyssus nasutus Hirst, 1925

Hirstionyssus callosciurus Bregetova & Grokhovskaya 1961

Hirstionyssus tamiopsis Wang, 1962

Ichoronyssus miniopterus (Womersley. 1957)

,, tieni (Grokhovskaya & Nguyn-Xuan-Hoe, 1961)

Neolaelaps magnistigmatus (Vitzthum, 1918)

Ornithonyssus bacoti (Hirst, 1919)

,, bursa (Berlese, 1888)

,, sylviarum (Canestrini & Fanzago, 1877)

Pellonyssus biscutatus (Hirst, 1921)

,, passeri Clark & Yunker, 1856

,, reedi (Zumpt & Patherson, 1952)

,, trachyphoni Till, 1963

,, zosteropus Till, 1963

II. Family HAEMOGAMASIDAE Oudemans, 1926

Eulaelaps stabularis (Koch, 1836)

III. Family LAELAPTIDAE Berlese, 1892

Androlaelaps hermaphroditus (Berlese, 1887)

Haemolaelaps diversichaetutus Grochovskaya & Nguyn-Xuan-Hoe, 1961

,, nadchatrami Baker, Traub & Evan, 1962

,, traubi (Strandtmann, 1948)

Laelaps (Laelaps) flagellifer Domrow, 1962

,, ,, myonyssognathus Grochovskaya & Nguyn-Xuan-Hoe, 1961

,, ,, nobilis Delfinado, 1960

,, ,, nuttalli Hirst, 1915

,, ,, turkestanicus Lange, 1955

,, ,, wasselli Domrow, 1958

Laelaps (Echinolaelaps aingworthae Strandtmann & Mitchell, 1963

,, ,, echidinus Berlese, 1887

,, ,, insignis Delfinado, 1960

,, ,, mercedae Strandtmann & Mitchell, 1963

,, ,, sanguisugus Vitzthum, 1926

,, ,, sculpteratus Vitzthum, 1926

,, ,, traubi Domrow, 1962

Longolaelaps longulus Vitzthum, 1926

,, whartoni Drummond & Baker, 1960

Rhyzolaelaps inaequipilis Bregetova & Grokhovskaya, 1961

Tricolaelaps comatus Vitzthum, 1926

IV. Family RHINONYSSIDAE Troussart, 1895

Rhinoecius bisetosus Strandtmann, 1952

V. Family SPINTURNICIDAE Oudemans, 1901

Ancystropus zeleborii Kolenati, 1956

Meristaspis lateralis (Kolenati, 1956)

Paraperiglishrus rhindophinus (Koch, 1841)

Spinturnix psi (Kolenati, 1856)

VI. Family CHEYLETIDAE

Chelacaropsis moorei Baker, 1949
Chelonotus selinirhynchus Berlese, 1893
Cheyleytus fortis Oudemans: 1 04
Neocheyletiella chanayi Berlese & Trouessart, 1880
Nihelia quinta Domrow & Baker, 1 63
,, calarata Domrow & Baker, 1 60

Tests on repellents against chiggers.

Tests were conducted with lindane-and carbaryl treated trousers against the chigger, Leptotrombidium akamushi. This mite was collected in small numbers from a grassy area at Kao Yai, south of Pak Chong. Mites were exposed in test cells on the treated garments. Trousers that were treated with lindane at 0.2 gm/ft and rinsed for 5 hours caused complete inactivation of chiggers within 30 minutes. Trousers treated with carbaryl at 2gm/ft caused complete inactivation in 10 minutes after they had been rinsed 4 hours but failed to cause complete knockdown within 15-minute test period after they had been rinsed 5 hours.

Evaluation of repellent against terrestrial leeches.

Two series of tests were also conducted with repellents against terrestrial leeches at Kao Yai Forest south of Pak Chong during August and September, 1966. Tropical fatigue trousers treated with lindane at 0.2 gm/ft or with carbaryl at 1.0 gm/ft were tested for effectiveness in repelling leeches when worn by Thai subjects along a leech infested forest trail. Some leeches stayed on the trousers treated with lindane, but others dropped off; all leeches dropped off the carbaryl-trated trousers. On untreated control trousers, the leeches acted about the same as on those treated with lindane. All treated and untreated trousers were rinsed by wading for 17 minutes in a mountain stream. The leeches would not crawl on the wet area of either the lindane-or carbaryl-treated trousers, but crawled on the control trousers. After the washing test, the trousers were dried and tested a week later. Some leeches crawled on the dried rinsed area of the lindane-treated trousers but not on the unrinsed area; the carbaryl-treated trousers repelled the leeches

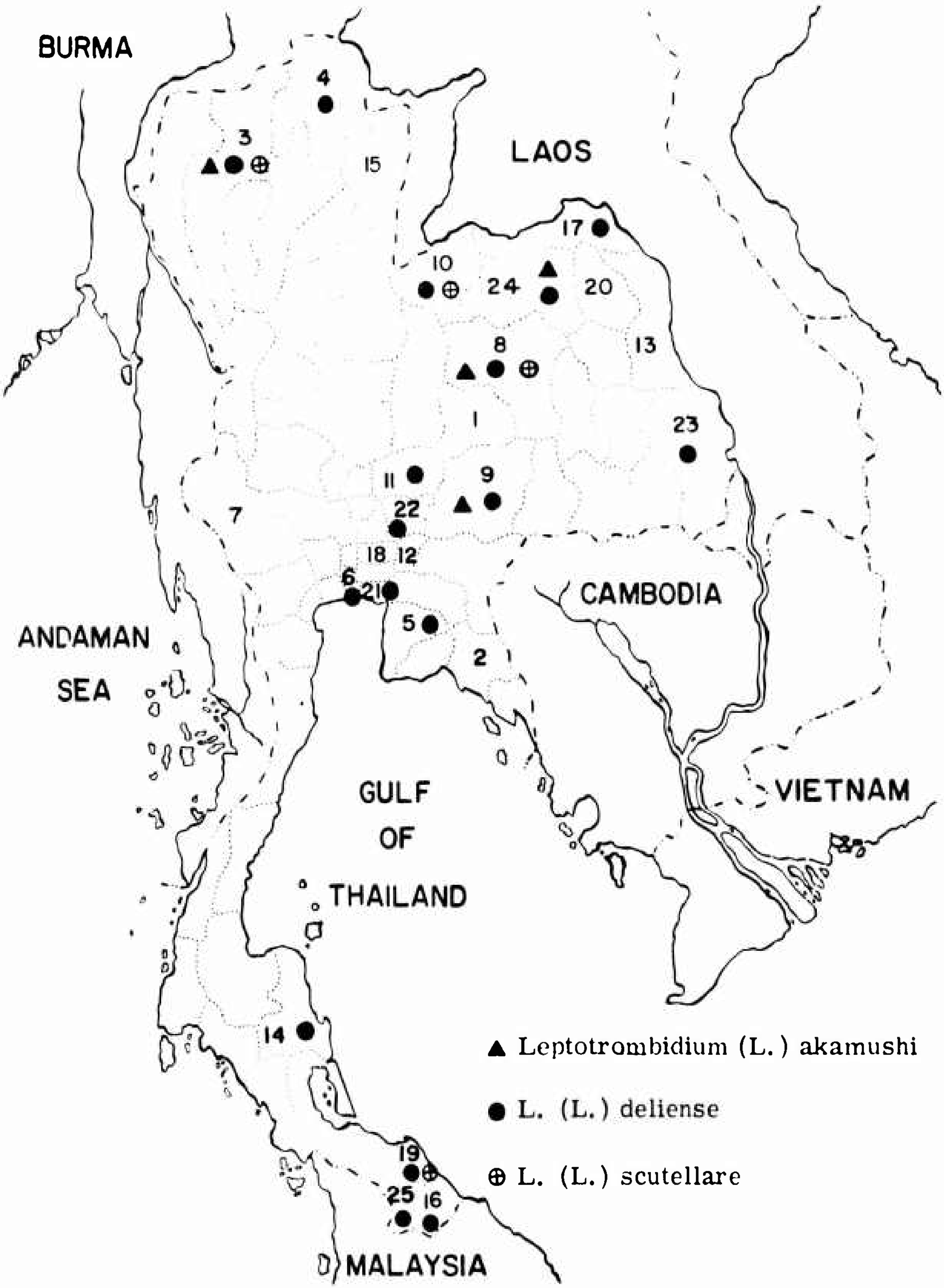
Summary: A host-parasite list of the chiggers (Trombiculidae) of Thailand has been completed. Among the chiggers examined during this period were eight species not previously known to occur in Thailand, and fourteen new species of the Genus Leptotrombidium which are being described and prepared for publication. A list of the known species of mites, other than chiggers, of Thailand was also prepared. This list includes a total of 48 species thus far known from this country. Tests conducted on lindane-or carbaryl-treated tropical fatigues for repellency against chiggers and leeches showed that carbaryl was the most effective repellent.

Publications:

1, Lakshana, P. A new species of trombiculid mite infesting scorpions in Thailand (Acarina, Trombiculidae). J. Med. Ent., 3(3-4): 258-260. (1966).

2. Traub, R & P. Lakshana. Some chiggers of the subgenus Leptotrombidium from Thailand, with descriptions of new species (Acarina, Trombiculidae). J. Med. Ent., 3(3-4: 271-296 (1 66.)

KNOWN DISTRIBUTION OF VECTOR SPECIES OF CHIGGERS IN THAILAND



SEATO MEDICAL RESEARCH STUDY ON ENTEROVIRUSES

Coordinator: Philip K. Russell, LTC MC

Principal Investigators: Rapin Snithbhan, M.D.
Chaninthorn Suwongse, M.D.

Associate Investigators: Dumrong Chiewsilp, M.D.
Pethai Mansuwan, M.D.
Chaiyan K. Sanyakorn, M.D.

Period of Report: 1 April 1966 31 March 1967

General Information:

No major research program on enteroviruses was carried out during the period of this report. However diagnostic studies on cases of central nervous system diseases indicated that poliovirus infection continues to be significant cause of meningitis and paralytic disease in Thailand. Eleven strains of type-1 poliovirus were isolated from 19 cases (8 from Bangkok and 5 from Korat) during 1966.

A serologic survey was done in February 1967 to determine the rate of acquisition of neutralizing antibody to polioviruses among children in Bangkok. Sera were collected from a random sample of children in the Makasan area of Bangkok. The 226 sera were a representative sample, the children under 15 years of age residing in the district. An additional 243 sera were collected from out-patients at the Children's Hospital. This second group was not a random sample. Neutralizing antibody was measured by testing heat inactivated (56°C, 30 min) serum diluted 1:10 against approximately 100 $TCID_{50}$ of each poliovirus serotype in a metabolic inhibition test using BS-C-1 cell culture. Tests were done in triplicate in plastic plates.

When testing was completed it was found that there were no differences in prevalence of antibody by age group between the two groups The combined results are summarized in Tables 1 and 2. In the age group under 2 years type-1 poliovirus antibody was significantly more prevalent than type-3. In the older groups the differences between types are minimal. Over 50% of children above the age of 5 have antibody to all three serotypes.

Table 1. Prevalence of polio neutralizing antibody by agent among children in Bangkok, February 1967

Age Group	Number Tested	Number with Antibody (%)		
		Type-1	Type-2	Type-3
<1	24	6 (25%)	6 (25%)	1 (4%)
1-3	131	82 (62%)	56 (42%)	36 (27%)
3 5	141	113 (80%)	101 (71%)	95 (69%)
5 7	77	68 (88%)	62 (80%)	54 (70%)
7-9	23	21 (91%)	23 (100%)	19 (83%)
9-11	22	22 (100%)	22 (100%)	19 (86%)
11-13	22	11 (86%)	22 (100%)	19 (86%)
13-15	29	23 (80%)	28 (96%)	25 (86%)

Table 2. Accumulation of neutralizing antibodies to 3 types of poliovirus with age among children in Bangkok.

Age Group	Percent with Antidody to:			
	None	1 Type	2 Types	3 Types
<1	50	46	4	0
1-3	18	40	30	12
3-5	3	21	36	44
5-7	5	11	27	57
7-9	0	4	17	80
9-11	0	0	13	87
11-13	0	9	9	82
13-15	0	5	9	86

SEATO Medical Research Study On

Eosinophilic Meningoencephalitis In Man

Coordinator: LTC. David M. Robinson, MC, USA

Principal Investigator: Major Sompone Punyagupta, MC, RTA*
CPT. James R. Crook, Ph.D.

Associate Investigators: Major Pipat Juttijudata, MC, RTA*
Thanongsak Bunnag, M.D.
Mr. Kamnird Supanwong, B.Sc.
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Mr. Chanin Pangmuangdee
Mr. Umpol NaNakorn
Mr. Prasit Limtrakul

Period of Report: 1 April 1966 - 31 March 1967

The studies so far completed show that eosinophilic meningitis is the most common parasitic disease of the central nervous system known in Thailand. It is a disease which is due to human ingestion of infective third stage larvae of A. cantonensis. Analysis of the more than 500 cases collected by Major Sompone suggests that the eating of raw Pila snails is the most common route of infection. However observations by Capt. Crook clearly show that infected pulmonate snails, upon drowning may shed into the water, larvae which are infective for animals and presumably man. Further, it has been shown that the standard chemical means of purifying water in the field will leave many of the shed larvae still infective. Thus the disease is already a problem among those who eat raw food and could possibly become a major problem to an army operating in the field, exposed to contaminated water.

A less common form of eosinophilic meningitis, the so called myeloencephalitic type, is described by Major Sompone. This differs from typical eosinophilic meningitis probably in etiology and certainly in its clinical manifestations and prognosis. Much more work needs to be done on this variety of the disease. The etiology is uncertain but at present it does not appear to be due to A. cantonensis. One of the great problems encountered in studying eosinophilic meningitis in whatever form, is the relative paucity of autopsy material in which an identifiable parasite can be found.

* General and Tropical Medicine Department, Pra-Mongkutkal General Hospital.

1. Title:	Studies on the Animal-Parasite Phase of Angiostrongylus cantonensis in Thailand. (Intermediate Host Studies; Definitive Host Studies)
Principal Investigator	Captain James R. Crook, Ph.D.
Associate Investigators:	SFC Samuel E. Fulton, AMEDS Mr. Kamnird Supanwong, B.Sc.
Period of Report:	1 April 1966 - 31 March 1967

OBJECTIVE: The objectives of this study are to evaluate potential, intermediate and definitive host species of Angiostrongylus cantonensis seasonally, in distinct areas of Thailand with regard to frequency of natural infection, ecology, distribution and the possible methods of either direct or indirect passage of the parasite to man.

DESCRIPTION: The areas selected for study are Bangkok-Dhonburi near the mouth of the Chao Phya River; Nakornrajsrima (Korat), Ubol and Udorn in the Northeast; Chiengmai, Sukothai and Nakhon Sawan in the North; Kanchanaburi and small villages up the Khwae Rivers in the West; Narathiwat, Yala, Haad Yai, Trang, Nakhon Sri Thammarat, Krabi, Ranong, Chumphon and Prachuab Khiri Khan in the South Peninsula; and Trat, Chanthaburi and Rayong in the Southeast. Conduct of the study was divided into two efforts. One effort concerned the definitive hosts, the second concerned the intermediate hosts. Collections of definitive host mammals were conducted by transect live trapping through habitats offering suitable conditions for both definitive and intermediate hosts. Rodents so obtained were dissected and examined for the presence of adult A. cantonensis worms in the pulmonary arteries. The remainder of the rodent was used in connection with other laboratory projects. The infected mammals were recorded as noted in previous reports. Intermediate host studies were begun along the above mentioned transects through comprehensive gastropod collections to approximately five meters on either side of the transect along its length. The resulting gastropod material was recorded, as previously outlined, and shipped to the Bangkok laboratory for processing. The individual species were identified and grouped in aquaria. Prior to examination for A. cantonensis third state larvae, the gastropods were examined for trematode intermediate stages by the Medical Malacology staff. The individual gastropods were then homogenized, digested and examined for the presence of A. cantonensis third stage larvae. All resulting metastrongylid larvae were fed by stomach tube into laboratory white Rattus norvegicus, WRAIR Strain. In 21 days the rat was sacrificed and the brain examined for the presence of fifth stage A. cantonensis larvae. Only after this definite proof, was a snail listed as positive. Natural epidemiological observations were conducted in the field during all studies to include possible gastropod-mammal and gastropod-human associations.

PROGRESS: South Peninsular Thailand was the first area studied during the period covered by this report. Throughout this generally tropical monsoon climatic area, a brief period, approximately from the last of February to mid April, is comparatively dry. To investigate the negative results obtained during the previous year between October and December, a comprehensive study was carried out during the relatively dry period. This season was chosen for ecological considerations. The dry period would allow rodent fecal material to collect in quantity and, provided there was some infection, increase the chance that suitable coprophagic

snails would come into contact with the material and become infected. Near the end of the dry period, the optimum conditions existed for a complete cycle of the parasite. The coprophagous gastropods had an increased opportunity for infection of sufficient duration to be infective. The possibility of finding mammals recently infected by eating infective snails, was correspondingly better. The remainder of the year the rains are of such a profuse nature as to prohibit the accumulation of infective fecal matter, allowing a minimum chance for gastropod infection and life cycle accomplishment. The study of those locations mentioned in the "Description" yielded 1,213 mammals of seven species. Twenty five positive Rattus norvegicus were found and one each Rattus rattus and Rattus exulans. Simultaneously 2,192 gastropods were collected. Examinations revealed 16 definitely positive Achatina fulica and four possible positives. The latter were metastrongylid larvae recovered dead which could not be fed to rats for proof of the species. Previous findings support the assumption that the dead larvae were A. cantonensis. This finding of only 20 infected gastropods, seemingly insignificant, serves to nullify the previous indication of a natural infection void in the Southern Peninsula of Thailand.

The positive findings in the south were so widely scattered that the infection, at the present time, can be regarded as insignificant. The finding of the infection only in A. fulica, an introduced species, and not in snails proven to be natural intermediate hosts elsewhere, indicates the possibility that the parasite was introduced into the peninsular region with A. fulica in late 1941. The low infection level presently indicated could be due to an interrelationship between the climatic interference in the life cycle and the present low level of the scattered A. fulica populations.

Studies in the northeast were conducted in the listed locations at the end of the hot season, May-June 1966, resulting in 327 rodents collected. Of that number, six were found to be positive. During the same period of time, 1,893 gastropods were collected with no positive isolates. The severe conditions of the Northeast hot season resulted in the majority of gastropods being collected from sub-surface or deep sub-humus layers in a state of estivation. This condition appears to have existed for at least one month. The estivating factor demonstrates climatic influence on gastropod vagility reducing the chances of infection to a minimum during this season.

Studies in the north were conducted at the listed sites during both the hot and cold seasons. During the hot season, April-June 1966, 241 mammals were collected of which 11 were positive. Among the 1,360 gastropods collected there were no positive isolates. These findings are commensurate with the findings in the northeast under the same climatic influences and are judged to be due to the same ecological factors. Mammal collections in the cold season totalled 541 animals of which 35 were positive. Ecological conditions, primarily moisture, were more favorable for pulmonate gastropod activity and infection as indicated by the result of eight positive and three probable positive isolates from 1,774 gastropods. The positive intermediate host fluctuation is notable in view of the relatively stable mammal infection rate.

Investigations in west Thailand commenced on 4 August 1966 during the rainy season. Among the 709 mammals collected, 59 were infected with A. cantonensis. The species Bandicota bengalensis accounted for all of the positive findings. Quantula, striata, a new intermediate host, was found to be the only positive gastropod among the seven species examined during that season. Dry season collections in the area commenced on 1 December 1966. Live trapping yielded 676 mammals of which 66 were positive. With one exception, R. rattus, the positive animals were B. bengalensis. An unseasonal downpour brought large numbers of slugs (Veronicella sp.) to the near surface of the jungle litter and, among other gastropods, 930 of the slugs were collected. Approximately eight percent of the slugs were positive as well as the consistent one to two percent infection in Q. striata. The slugs had been collected in small numbers during the rainy season, however, none were positive then.

Studies in southeast Thailand were begun during the rainy season, September 1966. The region was divided into two ecologic sectors one with approximately 4,000 mm of rain per year in Trad and Chanthaburi, and the second with approximately 1,500 mm of rain per year in Rayong. In the former sector, 1,415

mammals were collected yielding 121 positives. Simultaneous gastropod collections yielded 1,741 specimens. Both Hemiplecta distincta and A. fulica were positive with the latter species almost 25% infected. Dry season studies in the 4,000 mm rainfall sector yielded 1,043 mammals of which 75 were positive. The reason, January and February 1967, was extremely dry and the 672 gastropods collected required considerable effort. Again, A. fulica was infected but the prevalence had fallen to about 15%. No other species were positive in this collection. Rayong, the 1,500 mm rainfall sector, yielded 363 mammals during the October 1966 rainy season work. In spite of comprehensive coverage there were no positive isolates. Gastropod collections included two known intermediate hosts; however, no positive material was found. It is noted that this rainy season was, according to climatological data over a 30 year period, "average" in all measured respects. The climate is similar to that of the Ubol area in spite of Rayong's proximity to the sea. The resulting impact on the mammal population is wide dispersal without indication of notable concentrations of colonies. This situation, only slightly condensed, is exhibited in the town of Rayong itself. Under the present conditions it is questionable whether or not a significant population of A. cantonensis can be supported in the natural host populations. Due to the comprehensive nature of the first study in this area and the fact that the dry season would offer even less opportunity for finding the parasite and the firm indication, based on two years experience, that no meaningful result could come from such an expenditure of personnel and money, no further seasonal work was done in this 1,500 mm rainfall sector of Southeast Thailand.

Collections in selected localities of Bangkok and Dhonburi were continued in such a manner as to monitor the level of the parasite population and determine the cause of fluctuations should such occur. Seasonal variations were observed as in previous years. A primary factor in the parasitic population fluctuations was the vagility of the intermediate hosts which was most influenced by seasonal moisture and temperatures. The definitive host infection rate, as in previous reports, fluctuated between approximately 18 and 6 precent with the higher figure during the late rainy season and the lower at the end of the hot season. During this study, a new intermediate host, Melanoides tuberculata, was found.

Studies on larval shedding from drowned intermediate host gastropods were emphasized. Although these studies are not complete, it is clear that a drowned pulmonate gastropod will shed up to 33% of its infecting larval burden if it has had an A. cantonensis infection of sufficient duration and there is a large larval burden. Due to the fact that the only gastropods found to be of importance in the life cycle of A. cantonensis in Thailand are pulmonate, this response to drowning assumes important proportions. Observations of proven intermediate host gastropods contaminating culinary water, reported at the Second Medical Conference on Parasitic Diseases, March 7-11 1966, further added importance to this shedding response. Experiments to determine the effect of chemical water treatment on the infectivity of shed larvae were conducted in view of the indicated epidemiological importance of water contamination. Chlorine treatment was of no measurable value, Iodine water purification tablets, routinely issued by the U.S. military, were used as directed for extreme water pollution and resulted in the killing of about 66% of the larvae. It is noted, in spite of the 66% control, the remaining approximately 33% were infective as demonstrated by rat inoculation. Filtration through coarse material such as sand removed all larvae from the water.

SUMMARY: Collections continued throughout this year with emphasis on the ecological and epidemiological conditions prevailing in each major region of Thailand. A light infection rate was again demonstrated in the Northeast. The infection rate in the north also remained much as it had been described during the previous year. Studies in the west, conducted for the first time, revealed a high rate of infection and a previously unreported intermediate host. Studies in the south revealed a few positive animals indicating very small pockets of infection generally corresponding to small isolated populations of A. fulica. The latter species was introduced by Japanese occupation forces between 1941 and 1945. Local residents in the South Peninsula state that the snails were brought from Taiwan, the location of the first reported human case of infection by A. cantonensis. The possibility that the observed infection among mammals in the south was imported with the snails is presented for consideration. Studies in the southeast conducted for the first time, revealed very high levels of infection in the high rainfall areas of Trat and Chanthaburi; however, no infection was found

in the dry region of Rayong. Studies concerning the infection levels in the Bangkok-Dhonburi area continued with seasonal fluctuations commensurate with earlier observations. The observed fluctuations were due to the seasonal influences on pulmonate gastropod intermediate hosts. During this year's examinations, no fresh or brackish water snails were found to be infected in spite of the thousands processed from areas exhibiting high infection rates among other gastropods.

The study emphasized the importance of intra-area evaluation of all ecological, life cycle, and epidemiological factors due to the fact that each region exhibited some degree of life cycle difference. The main difference in life cycles concerns the intermediate hosts involved in a given area and the influence of the various ecological conditions on those intermediate hosts. No two regions exhibited the same collection of proven intermediate hosts and since it is from a suitable intermediate host that mammalian infective third stage larvae must originate, epidemiological hypotheses obviously must be formulated from findings in the area in question.

Studies concerning the larval shedding from drowned pulmonate snails continue to emphasize the importance of this phenomenon in epidemiological considerations. This assumes proper perspective when it is noted that to this date, only pulmonate land snails have been shown to be of importance in the natural life cycle of the parasite throughout the Kingdom of Thailand. Further importance is directed to this to situation with the realization that recognized chemical treatment does not kill all of the larvae in the water and those remaining are infective.

(Annual report April 1966 — March 1967)

STUDY REPORT

Title:	Clinical Manifestations and Epidemiological Studies of Eosinophilic Meningoencephalitis in Man.
Principal Investigator:	Sompone Punyagupta Maj, MC. RTA
Associate Investigators:	Pipat Juttijudata Maj. MC. RTA Thanongsak Bunnag M.D.
Assistant Investigators:	Chanin Pangmuangdee Umpol NaNakorn Prasit Limtrakul

OBJECTIVES: To continue the complete epidemiological and clinical studies and laboratory evaluations on cases of eosinophilic meningoencephalitis in Thai subjects and perform such special animal investigations as are indicated. Based on last year's study, it is certain that this disease is the most common and important parasitic disease of the central nervous system in Thailand. Many aspects of the disease are still not known.

DESCRIPTION: During the period, 4 provinces were studied (namely Bangkok, Korat, Ubol and Pranchinburi). The first two provinces are the endemic areas of so called "typical type" of eosinophilic meningitis while "myeloencephalitis type" is commonly seen in the latter two provinces. In every proven positive case, a complete epidemiological evaluation, and clinical and laboratory investigations were performed. In all fatal cases, efforts were made to obtain autopsies in order to study the pathological changes.

Research studies in the laboratory were done to confirm the importance of Pila snails as the main source of human infection. Experimental animal studies were done to compare the local *Angiostrongylus cantonensis* to the Honolulu strain. The studies on the biochemical changes in the cerebrospinal fluid of eosinophilic meningitis cases were made in cooperation with Cpt., R.A. Rasmusser, Dept of Clinical Chemistry Division of Biochemistry, WRAIR.

PROGRESS: During this year, 166 cases of eosinophilic meningitis were studied, which makes a total of 512 cases for the two year study. Of these 166 cases, 150 belong to the "typical" Eosinophilic Meningitis and 16 are classified as myeloencephalitis.

TABLE 1

I. Epidemiology: Eosinophilic meningitis has been reported in 33 provinces; 18 central, 13 in the northeast and one each in the north and south.

TABLE 2

1. Age and sex distribution. The disease occurred at all ages but is most prevalent in the second and third decades. The youngest in this study was a two year old girl. Males were involved twice as often as females.

2. Seasonal Variations. The number of cases diagnosed and the rainfall registered in three provinces (Bangkok, Korat and Ubol) are presented in graph I. The rainfall pattern in these areas was wore or less the same, starting in April and continuing through November. In Bangkok the disease, mainly typical Eosinophilic Meningitis, was most prevalent during July and December. In Korat, however, the disease, also typical Eosinophilic Meningitis, occurred as a year round disease but the peaks were found to rise later than Bangkok, In Ubol, where the majority of cases were myeloencephalitis type the peak of the disease seems to occur sooner than in Bangkok. In these areas, there seems to be a correlation between the availability of the *Pila* snails and the peak of the disease. The snails are amphibious and they will start breeding soon after the rainy season. It takes a few months for snails to grow big enough to be eaten. They are abundant everywhere in the rainy season. In the dry season they are found only occasionally in ponds. Another explanation for the seasonal variation of this disease is the ease with which patients can come to the hospital. This is important in Korat which is a big province and has poor communications during the rainy period. In addition the rainy season is the harvesting time and unless they are very sick, patients will try not to come to the hospital.

3. Incubation period. The incubation period was not known in about 35 percent of cases of Eosinophilic Meningitis, because of either multiple possible sources of infection or an uncertain history. However in the cases who gave a definite history of ingesting only a single raw food within one month prior to the onset of symptoms, the incubation period varies from as short as one day to as long as 47 days. The majority of cases, however, have an incubation period of 3 to 20 days.

The incubation period can be determined more reliably by studying patients who came in groups having once joined in eating the same dish of raw food and having become sick within 1-2 weeks of each other. There were 34 such groups and a total of 132 cases. The incubation period varies from 3-36 days with the average of 16.5 days.

4. Sources of Human Infection. Eighty-five percent of typical EM cases gave a definite history of eating raw food only once during the one month prior to the onset of symptoms. Out of these, 84% gave a definite history of eating raw *Pila* snails. Fresh water shrimp and fresh water fish were eaten by 8.2 and 3.5 percent respectively. The rest ate other snails, crabs and beef. In myeloencephalitis type. 73% gave a definite history of eating raw food. 50% ate *Pila* snails, 26% fresh water shrimp, 7% water fish, 11% raw beef, Of the 34 "groups" of patients, all gave a definite history of eating only raw *Pila* together.

TABLE 3

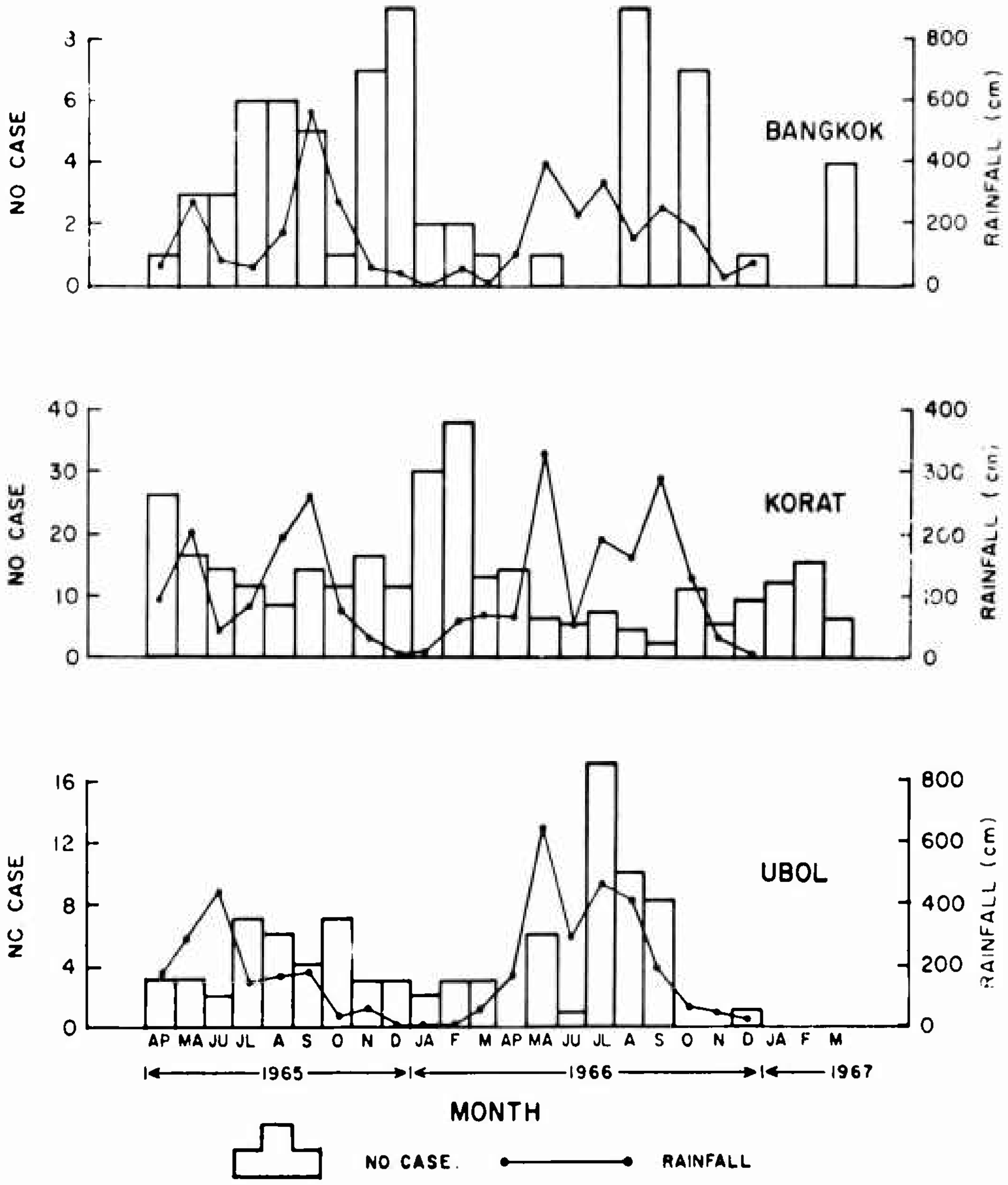
To confirm this epidemiological fact, *Pila* snails were collected from those areas where patients obtained the snails for eating. They were carefully studied for the presence of any parasites. *Pila* snails from 10 out of 27 areas examined were found to harbour infective *A. cantonensis* larvae, 16% of 236 s examined were positive. The rate of infection in snails in each area varies from 1.8 to 72.7 percent. number of infective third stage larvae found in positive snails varied from 1 to 732 with an average of No infective *Gnathostoma* larvae were found.

TABLE 4

II Clinical Manifestations:

There were 478 cases of typical EM and 54 cases of the myeloencephalitis type. Headache is a constant finding in the typical EM but not in myeloencephalitis. Limb paralysis was not found in typical EM

GRAPH I SEASONAL VARIATION OF EOSINOPHILIC MENINGOENCEPHALITIS
AND
THE RAINFALL IN BANGKOK, KORAT AND UBOL IN 1965-1967



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while it was a constant finding in myeloencephalitis. Vision impairment occurred in both types. 11% in typical EM and 9% in myeloencephalitis. Facial Paralysis occurs in about 4% in both types. 2% of typical EM cases and 6% of myeloencephalitis cases experienced lateral rectus paralysis. Eye edema was note in 6% myeloencephalitis cases and only 0.8% in typical EM cases. Death occurred in 13 cases or 22.2% of myeloencephalitis and 6 cases of typical EM or 1.2%.

TABLE 5, 6

III. Laboratory Studies

A leucocytosis over 10,000/cumm. was found in 52% of both types. Eosinophilia in the peripheral blood of over 10% was found in 72% of typical EM cases while only 11% of myelitis case showed this. The times of the highest cell counts in the spinal fluid fell within the first two weeks in 81.3%. The highest cell count in 83.8% of cases was between 200-4999 cells/cm,mm. 81% of typical EM and 64% of the myelitis type had an eosinophilia in the CSF of over 25%. Protein content of the CSF was over 50 mg percent in 72% to typical EM and 78% of myelitis cases. There have been no significant changes in the sugar and chloride content of the CSF. The opening pressure of the CSF during the acute stage was found to be increased in 45% of typical EM and 28% of myeloencephalitis cases.

IV. Special CSF Study

The spinal fluids of 44 cases were sent to Capt. Rasmussen at WRAIR for the special electrophoresis and enzyme studies. The following interesting point were noted.

1. 91% of cases studied had an abnormally high gamma globulin in the CSF.

2. LDH enzyme in the CSF was abnormal in 72% of cases particularly in the 5th and 4th fraction.

3. There is an interesting reverse relationship in enzyme GOT between the sera and the CSF in some instances: that is the GOT level in the CSF was higher than its level in the serum.

4. Using an electrophoretic technic with higher resolving power remarkable multiple bands in the gamma globulin fraction of the CSF were noted while no such bands were found in the sera.

Further studies are being done.

V. Pathology

Out of 13 deaths in the myeloencephalitis type only 6 autopsies were performed. No etiologic agent was found. The detailed pathological findings will be presented elsewhere. However Dr. Prasan Tangchai, a neuropathologist at Chulalongkorn Hospital, reported finding A. cantonensis in the brains of two Thai patients who died of meningitis. Comparing his histologic findings and the findings in the brains of our patients who died with the myelitis type of disease, it seems that the etiologic agent in our myeloencephalitis cases is not A. cantonensis. Histologically one may find microscopic necrotic, hemorrhagic areas in cases typical E.M. and grossly visible tracts in the myelitis type.

VI. Experimental Studies

I. Comparative studies of Thai and Honolulu Strains of A. cantonensis in Rats and Hamsters.

The infective larvae of local A. cantonensis (Nakornsawan) and Honolulu strain (Dr. Rosen's Lab) were given to white rats and hamsters. These animals were sacrificed at intervals and were examined for parasites. It was found that the Honolulu strain reached the heart and lungs 4 days sooner than the local

strain (29th vs. 33 day). It also appears that after the 10th day the local strain seems to have grown faster and bigger than Honolulu strain. The same findings are seen in the Hamsters. There have been no differences in the rate of larvae recovery between these two strains.

#2 Studies on Pila snails as intermediate hosts.

First stage larvae of local A. cantonensis were fed into naturally collected P. ampullacea and also to laboratory bred P. polita. In naturally collected snails, 25-42 percent of the larvae were recovered during the first week after infection; thereafter the percentage of larvae recovered fell. The third stage infective larvae were first recognized on the 21st day. On 47th day after infection only 3% of larvae were recovered from the snail. In the young, small (about 1 1/2 cm in size) laboratory bred P. polita the recovery rate in the first week was much higher, 60-93 percent. However the recovery rate fell thereafter to about 20% and remained at this level to the 47th day. The infective larvae were found in the foot of the snail. This experiment confirms that Pila snails are an intermediate host of A. cantonensis. The younger and smaller snails seem to be a much better host. The larvae can develope to the infective stage in as little as 18 days. There has been no explanation yet for the spontaneous reduction in the number of infective larvae in snails.

SUMMARY:

From this evidence certain conclusions may be drawn:

1. Eosinophilic meningitis is a significant disease and an important public health problem in the studied areas. The disease occurs at all ages but is more common in the second and third decades. It involves males twice as often as females. It can be found at any time of the year but the highest attack rate is in the rainy season.

2. Two distinct types of eosinophilic meningitis were recognized; one was called typical eosinophilic meningitis and another one was myeloencephalitis. Impairment of vision is an important complication in the disease.

3. The mortality rate was 1.2% in typical form and 12.9% in the myeloencephalitis form.

4. A. cantonensis is a causative agent in typical eosinophilic meningitis. No agent can be found as a cause of the myeloencephalitis type and there is evidence it is not A. cantonensis.

5. Eating raw Pila snails containing the infective larvae of Angiostrongylus cantonensis is the main source of typical E.M. in man, in Thailand. The consumption of other raw animals such as fresh water shrimp or fish may be of significance. In about 15% of the cases, no single source or route infection can be found.

6. The number of A. cantonensis larvae needed to produce the clinical disease in man probably varies a great deal, from as few as 1 or 2 to as many as hundreds. The difference in the number of larvae ingested may account for the wide range of the incubation periods as well as the varying severity and clinical manifestations.

TABLE 1:

Eosinophilic Meningoencephalitis Studied During April 1966 March 1967

Province	First year	Second year	Total
Bangkok	46	22	68
Korat	208	96	304
Ubol	46	43	89
Udorn	31	—	31
Prachinburi	16	4	20
Total	347	165	512

TABLE 2

List of Provinces where Eosinophilic Meningoencephalitis was reported.

CENTRAL THAILAND

Bangkok and Dhonburi
Chacherngsao
Pathoomthani
Nakornnayoke
Saraburi
Singburi
Chainat
Nakornsawan
Pisnuloke

Samutprakarn
Prachinburi
Ayudhaya
Suphanburi
Angthong
Lopburi
Uthaithani
Kampangpetch
Sukothai

NORTHEAST

Nakornrajsima
Srisakate
Chaiyaphoom
Mahasarakam
Kalasin
Nongkai
Nakornpanom

Bureerum
Ubol
Royed
Konkaen
Udorn
Sakolnakorn

NORTH

Chiengrai

SOUTH

Prachuabkirikan

TABLE 3

Number and Percent of 305 Patients with Eosinophilic Meningoencephalitis who consumed only one raw food within 4 weeks prior to the onset of first symptom according to 2 major types.

	Typical EM		Myeloencephalitis	
	Number	Percent	Number	Percent
Pila snails	237	84.+	13	50.+
Other snails	3	1.1	—	—
Small fresh water shrimp	21	7.5	7	26.9
Large fresh water shrimp	2	0.7	—	—
Small fresh water fish	9	3.2	1	3.8
Large fresh water fish	1	0.4	1	3.8
Crabs	1	0.4	1	3.8
Somfuk, Pla Som Raw Fish Dish	1	0,4	—	—
Koi Pla Ra Row Fish Dishes	4	1.4	3	11.5
Total	279		26	

TABLE 4

The Result on Examining Pila Snails From 27 Infective Areas For the Infective Larvae of A. cantonensis.

Location	No. Areas examined	No. Areas positive	Type of snails	No. snail examined	Percent of positive snail	No. larvae in each positive snali Minimum	Maximum	Mean
Bangkoo	4	2	P. polita	17	0			
			P. ampullacea	56	1.8	5	5	5
Korat	19	4	P. polita	105	8.5	1	12	2.8
Chaiyaphoom	2	2	P. palita	18	16.7	2	5	3.3
Lopburi	1	1	P. polita	7	14.2	2	2	2
Nakornsawan	1	1	P. scutata	33	72.7	1	732	107
Total	27	10	----	236	16.1	1 5	2 732	68.6

Table 5

Summary on the Clinical Manifestations of 532 Cases of Eosinophilic Meningitis in Thailand According to Major Clinical Types

Clinical Manifestations	Typical Eosinophilic Meningitis	Myeloencephalitis
Headache	98	74
Temperature over 37°C	38	68
Facial palsy	4	4
Lateral rectus paralysis	2	6
Vision impairment	11	9
Signs of meningeal irratation	23	31
Eye edema	0.4	6
Coma	0.8	6
Paralysis of limb		100
Xanthochromic spinal fluid	5	24
Mortality rate	1.2	22.2
Total Cases	478	54

TABLE 6

The difference between Typical Eosinophilic Meningoencephalitis

and Myeloencephalitis in 5 Provinces of Thailand.

	Typical Eosinophilic Meningoencephalits	Eosinophilic Myeloencephalitis
I. Epidemiology		
Location	Bangkok, Korat and Udorn	Ubol and Prachinburi
Seasonal Variations	3-4 Months after heavy rain or year round	Mid-rainy season
Suspected food	Mainly Pila snails	Uncertain
II. Clinical		
Severe Headache	Constant	Uncommon
Eye Edema	Rare	Common
Limb Paralysis	Not seen	Constant
Xanthochromia spinal fluid	Rare	Common
III. Mortality	Rare (1.2%)	High (13%)
IV. Pathology	Brain only No macroscopic parasitic tracts	Cord and brain hemorrhagic and necrotic macroscopic parasitic tracts
V. Etiologic Agent	Angiostrongylus Cantonensis	Unknown

SEATO Medical Research Studies on Gnathostomiasis in Thailand.

Co-ordinator: Professor Svasti Daengsvang, M.D.
Special Consultant to the Director.

Principal Investigator: Professor Svasti Daengsvang M.D.

Associate Investigator: Dr. Kampol Pecharanond M.D.*

Assistant Investigators: Miss Boonsiri Phrukoudom
Mr. Pichit Youngyi B.Sc.

Period of Report: 1 April 1966 to 31 March 19

General Information

Human and animal gnathostomiasis caused by Gnathostoma spinigerum is highly endemic in Thailand and appears to be on the increase. Recently, an autopsy done on a female patient who died of symptoms referable to the central nervous system at Thonburi Mental Disease Hospital showed damage done to her CNS by an adult male G. spinigerum. After World War II, many hundreds of human cases of gnathostomiasis were found in the Prefecture of Kyushu, Hanshu and Shikoku in Japan and the disease seems to be extending gradually in that country. Human cases of infection with G. spinigerum have been reported from many countries in Asia. Twenty species of Gnathostoma have been recorded in the literature although only 8 seem to be recognized at present as distinct species. Among them, only Gnathostoma spinigerum was reported as a cause of human gnathostomiasis in Thailand and other countries except for one case each from Japan and Canton and two cases from India which were infected with G. hispidum. Most of the human infections have been with mature male G. spinigerum found in subcutaneous, visceral organs and other tissues.

The life cycle of G. spinigerum and some methods of its spread and prevention had been worked out in this country before the Second World War. In the Annual Progress Report for 1 April 1965 to 31 March 1966 it was noted that aspects of its epidemiology, individual and community preventive measures, diagnosis, pathological changes and treatment have not been fully studied. The present studies on gnathostomiasis therefore aim to achieve solutions for the above-mentioned problems.

Recently, domestic pigs slaughtered in Bangkok, Nakornpathom, Rajaburi, Nakornsrithammarat and Phuket provinces were found infected with G. hispidum in their stomachs. This problem also should be given further consideration concerning the possibility of human infection with this species of which its transmission reported by Golovin (1956) in Russia to the final host (pig) was claimed to be experimentally possible either by drinking water containing the infected cyclops or by eating flesh of the reservoir hosts e.g. fish, amphibians or reptiles. (Helminthological Abstracts, Vol. 25, 265-266, 1956).

*Department of Parasitology, Faculty of Medicine Chulalongkorn Hospital.

Study Reports

Title:	Gnathostomiasis in Thailand
Principal Investigator:	Prof. Svasti Daengsvang, M.D.
Associate Investigator,	Dr. Kampol Pecharanond, M.D.*
Assistant Investigators:	Miss Boonsiri Phrukoudom Mr. Pichit Youngyi B.Sc.

Objective: The purpose of these studies is to determine the prevalence of Gnathostoma spinigerum among man and animals in Thailand and to carry out further clinical and epidemiological studies including individual and community preventive measures, methods of diagnosis, pathological changes of infected or ans and treatment of the disease. Several years before World War II, dogs and cats were shown to be natural or definitive hosts of the parasite in this country. Therefore tigers and leopards were found infected with adult worms in their stomachs. One leopard cat (Felis bengalensis) among 32 wild-caught carnivorous animals in Bangkok Dusit Zoo was found positive with presumably G. spinigerum ova in its stool as appeared in the last Annual Progress Report, 1 April 1965 to 31 March 1966. These domestic and wild animals therefore can act as reservoir hosts by passing eggs of the worm in their stools. The life cycle and method of transmission of this helminth were worked out in 1936, but many unsolved problems including epidemiological aspects, intermediate hosts and preventive measures remain to be further clarified.

Human cases of gnathostomiasis have been recognized in this country as due to only one species of Gnathostoma, that is G. spinigerum. The effect in man has been varied including lesions of respiratory and visceral organs, lesions of skin, eyes, mucous membranes, meninges and central nervous system damage in one instance. This parasitic helminth has been found in various parts of human body but pathological changes of the infected organs caused by the worm have not been fully clarified. There is, as yet, no effective chemotherapy; surgical removal of the worm from the infected organs was successful in only a few human cases in which non-vital organs were involved and the worm could be definitely located.

Description: The regional prevalence of human gnathostomiasis was investigated last year by collecting cases reported on prepared data-report forms from hospitals and personal communication with medical practitioners, health officers, hospital doctors and nurses as many as possible. This year parts of the Southern region of the country were visited by the team for obtaining more information about the prevalence of this disease in man from local workers and people. Further determination of the significance of animals that may ask as reservoir hosts of the disease was carried out by examination of stools or gastro-intestinal tracts of cats, dogs and other suspected domestic and wild-caught animals in Bangkok. Concurrently, studies to determine the natural animal sources of infection to definitive hosts and to man were continued from last year by examination of fresh-water fish and other animals. Much attention also was paid to examination of animal foods and domestic animals frequently eaten by definitive hosts. Experimental infection of many laboratory and other animals with the third-stage and second-stage larvae of the parasite was further undertaken to determine possible or potential second intermediate and paratenic hosts, to determine more

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susceptable animals than found in the previous year that could be properly used as laboratory hosts, to study detailed development and viability of the larvae in vivo and vitro, to determine the limitation of the number of the third-stage larvae after being fed repeatedly by paratenic host and the infectivity rate of the larvae in cats and dogs. Studies were also designed to determine pathological changes in detail of the infected organs and prenatal infection caused by the third-stage and second-stage larvae of the worm in non-pregnant and pregnant laboratory animals.

Progress: To further determine the prevalence of human gnathostomiasis in the Southern region, some selected areas of Provinces of Nakornsrithammarat, Phuket. Ranong and Prachuabkhirikhan were visited during the period. Many local medical practitioners, health officers, hospital doctors and nurses as well as some lay people were interviewed. Information provedea by the above-mentioned people indicated that some cases of the disease were diagnosed yearly but the symptoms were mild, mostly they suffered from off and on migratory swellings of the skin accompanied by a slight degree of pain and itching. Approximately 15-20 cases were found yearly in those four different provinces and usually some sought diagnosis and treatment from medical private practitioners and many were self-treated by coating the affected areas with indigenous medications. A few were removed from the skin by finger pressure. The incidence of Human gnathostomiasis appears to be higher in the Southern region and appears to be higher than given on prepared data-report forms subitted by the hospital authorities because cases of the disease were not sufficiently ill to seek for hospital assistance.

Due to the need for detailed information concerning the spread of G. spinigerum among animals and subsequently to man, the studies to determine the significance of animals acting as definite or reservoir hosts was continued from the previous year as appeared in Annual Progress Report for the year 1965-1966 with the co-operation and assistance of the Rabies Control Units of Bangok and Thonburi Municipalities, Provincial and Municipal Health Offices of the Southern Thailand including Nakornsrithammarat, Phuket, Ranong and Prachuabkhirikhan with following details:

In Bangkok area, of 4,509 dog stomachs 105 (2.3%) were found positive with adult male and female G. spinigerum during the year. It is interesting to note that dog stomachs at Bangkok and Thonburi Rabies Control Unit were found to be positive only during the months of June to January which is the rain and early part of dry seasons. Examinations of 1,276 dog stomachs during dry season of the year (February to May) were negative. Only 1 of 44 dog stomachs examined in February in the South at Nakornsrithammarat was positive with one immature G. spinigerum.

Of total 423 pig stomachs examined in 4 provinces of Southern region, 37 were found to be infected with G. doloresi and 7 with G. hispidum of which the details are as follows:

In Nakornsrithammarat, of 140 pig stomachs 6 were positive with G. hispidum and 18 with G. doloresi. (2 stomachs, one of a leopard cat and one of an otter were negative.)

In Phuket of 273 stomachs from pigs, 1 was positive with G. hispidum and 18 with G. doloresi.

In Ranong only 1 stomach of wild pig examined was positive with one adult female G. doloresi.

In Prachuabkhirikhan, all 9 pig stomachs were found negative.

The result of stool examinations of 127 cats (Bangkok 72, Ayuthaya 3, Nakornsrithammarat 16, Phuket 14, Ranong 9, Prachuabkhirikhan 13), 7 dogs (Nakornsrithammarat, Phuket, Ranong and Prachuabkhirikhan) and 18 wild-caught animals in Nakornsrithammarat were found to be negative.

In Summary 106 (2.3%) of 4,553 dogs examined were positive mostly in Bangkok area during rainy or wet season of the year but all 127 domestic cats and 20 wild-caught animals were negative.

Determination of natural infection of more fresh-water fish and other animals carrying the third-stage or infective larvae of G. spinigerum to human beings and definite hosts were also undertaken. Due to the regular supply in many months of the yea of dead snakes, made possible by the Science Division, National Red Cross Society special attention was paid to the study on seasonal variation of the incidence of this infection among them. These snakes were all obtained from provinces in central Thailand except one king cobra and one cobra from the South. In future if possible it is also planned to determine seasonal distribution of other animals acting as the source of infective larvae to man and animal especially in central Thailand which is the highly endemic area of the parasite. Concurrently, experimental feedings with third-stage larvae removed from second intermediate hosts as well as second-stage larvae or fully developed larvae in cyclops were carried out on many species of animals to determine possible or potential second intermediate or paratenic hosts of the parasite, what animals could be appropriately used as laboratory hosts and lastly more detailed studies of the development and viability of the larvae in vivo and invitro. All these animals were exumied for the third-stage or infective larvae in their flesh and other organs by using an electrical illumination apparatus and the identification of the larvae were then confirmed by microscopic examination. Examination for the natural infection with G. spinigerum third-stage larvae of many species of animals showed them encysted in the flesh and visceral organs of two more species of animals than previously recorded namely Ophicephalus lucius and Trimeresus gramineus (green pit viper). These animals were obtained from many provinces in central, southern and eastern regions of the country. The result of examination for natural infection of the infective larvae of the parasite during the period 1 April 1966 to 31 March 1967 showed that of 573 fresh-water fish examined 121 (21.5%) were found to be infected among which snakeheaded fish (Ophicephalus) showed still the highest rate of infection about 20%. Theincidence of infection of fresh-water fish found in last year was only 3.13% of which 68 (32.0%) of 212 snake-headed fish examined were positive. The maximum number of larvae in one infected Ophicephalus fish was 14 (49 last year). Of 255 snake-headed fish examined 111 (43.0%) were positive which is higher than that of the last year. At lower rates of infection were eel (Synbranchus bengalensis) 13.0% and cat-fish (Clarias Sup.) 2.7%. The species of various fish and other animals from which the infective larvae were recovered are listed in Table I. Fish and other animals from which larvae were not recovered by this year survey and of which more than ten specimens were examined are listed in Table II. In addition, the species of which only a few specimens were examined and negative are listed in Table III.

Among 44 amphibians examined, only 6 frogs of one species (Rana rugulosa) were positive. The result of examination of snakes obtained by the Bangkok Snake Farm of the National Red Cross Society from Samutprakarn, Angthong and Ayuthaya except one king cobra collected from Nakornsrithammarat and one cobra from Phuket was as follows: Of 3 king cobras (Naja hannah) 2 were positive with an average of 16 larvae and in 12 of 430 cobras found to be positive with an average of 248 larvae per animal. A single larvae was found in a green pit viper (Trimeresus gramineus) which is the first to be recorded in this snake. No larvae were recovered from 87 Vipera russelli, 39 Agkistrodon blomhoffi, 36 Bungarus fasciatus, 16 Boiga, one Python reticulatus and one Cylindromhis rufus. The monthly incidences of snakes found to be positive with the larvae seemed to be higher in the rainy season than other months of the year.

Table 1 Natural infection with G. spinigerum third-stage larvae No. positive/No. examined.

Species of animal	Bangkok	Thonburi	Samut prakarn	Cha-choeng-sao	Trat	Ayu-tha-ya	Kan-chana buri	Ang-thong	Sara-buri	Udon	Pheiburi	Pra-chuap-khiri-khan	Ra-nong	Nakhon-sitham-marat	Phuket
Ophicephalus striatus (Snake-headed fish) Large Small	1/8	 1/14	7/7 34/80	7/10 29/51		5/7		6/6 3/6			0/1 0/9		1/7	12/28	5/21
Ophicephalus micropeltes			1/1											2/8	
Ophicephalus lucius*			2/5											1/1	
Clarias macrocephalus	0/2		1/32	1/1							0/2	0/17	0/11	2/57	0/25
Synbranchus bengalensis (Eel)	0/4			1/2								0/1		1/9	
Rana rugulosa (Frog)	0/2	1/2	2/5						3/12	0/11					
Naja hannah (King cobra)						1/1		0/1						1/1	
Naja naja (cobra)	12/428				0/1										0/1
Trimeresus gramineus* (Green pit vipper)	1/5														
Bubulcus coromandus (Cattle egret)														1/3	
Corvus macrorhynchos (Crow)					2/2										
Anas plytyrhynchus domestica (Domestic duck)	0/2											0/1	0/1	1/1	
Bandicota indica (Bandicoot rat)	1/62						0/5							0/2	

* New species found infected.

Table II Animals Negative for G. spinigerum.
(10 and more specimens of each species examnied.)

Class	Species	Number
Crustacea	Paratelphus sexpunctatum	73*
	Palaemon potamiscus	13694
Pisces	Ompok bimaculatus	14*
	Trichopodus trichopterus	25*
	Anabus testudineus	18
	Notopterus notopterus	36
	Puntius gonionotus	11
	Tilapia mossombica	10
Reptilia	Vipera russelli	87
	Bungarus fasciatus	36
	Agkistrodon blomhoffi	39
	Boiga	16
	Leiolepis belliana belliana	17
	Calotes versicolor	22
Aves	Cynopterus brachyotis	215*
	Gallus gallus domesticus	11
Mammalia	Rattus exulans	51*
	Rattus rattus	169*
	Rattus norvegicus	164*
	Rattus rajah	16
	Mus famulus	10
	Tupaia glis	17*
	Sus scrofa domestica	900**

** Only 100 grams of fat and muscles from one hind leg and chest of each pig were examined through the kind assistance of Live-stock Trading Co-operation (Bangkok Pig Slaughter House).

* Represents collection from more than two provinces.

Table III Animals Negative for G. spinigerum
(Less than 10 specimens of each species examined)

Class	Species	Number
Pisces	Catopra siamensis	7
	Puntius orphoides	8
	Trichopsis vittatus	1
	Osteogeneiosus militaris	2
	Hampala macrolepidota	2
	Macrognathus aculeata	2
	Labiobarbus siamensis	2
	Bagroides macrocanthus	1
	Trichogaster pectoralis	1

Amphibia	Rana limnocharis limnocharis	8
	Bufo melanostictus	4
Reptilia	Python reticulatus	1
	Cylindromhis rufus	1
	Riopa herberti	2
	Varanus nebulosus	1
Aves	Arachuothera longirostra	5
	Phycnonotus blanfordi	4
	Maeronous guraris moltingadult	1
	Lonchura striata	3
	Longhura punctulata	4
	Paser montaneus	3
	Passer flaveolus	1
	Ploceus philippinus	9
	Strunus trislis	1
	Pycnomlus blangferdi	1
	Ardeola grayii	1
	Haliastur indus	4*
	Pycnonotus goiavier	2
	Rhipidura javanica	1
	Hypothymis azurea	1
	Dicunm cruentatum	1
	Trichastoma abbotti	1
	Dicrurus paradiseus	1
	Unidentified fish-eating bird	2
Mammalia	Rattus norvegicus var albinus	1
	Rattus niviventor	1
	Mus cervicolor	1
	Menetus berdmoici	1
	Suncus murinus	4

* Represents collection from more than two provinces.

Among birds examined, 1 of 3 cattle egrets (Bubulcus coromandus), 2 crows (Corvus macrorhynchos) and 1 of 5 domestic ducks (Anas platyrhynchus domestica) were found positive (Table 1.) Specimens of 21 other species were found negative as shown in Table II and III.

Only one of 69 bandicoot rats, mostly trapped in Bangkok was found positive. One thousand three hundred and thirty five mammals of several species were found to be negative as listed in Table II and III.

The animals collected from the central area of the country had a higher prevalence of infection with the third-stage larvae than those from the southern other areas.

In general, it is obvious that among animals found to be naturally infected with the larvae, snake-headed fish, eels and frogs showed higher incidence of infection than other species. The detailed findings of naturally infected animals are shown in Table I.

Experimental infection of certain animals with known numbers of the third-stage larvae of the parasite for determination of possible or potential second intermediate hosts as well as paratenic hosts showed that 23 species of animals in five classes had the larvae in liver, lung, heart, heart blood, kidney, pancreas, stomach, intestinal and esophageal walls, mesentery, omentum, subcutaneous tissue and flesh (Table IV). Of these experimentally infected animals, 8 species including Ophicephalus striatus (snake-headed fish), Clarias macrocephalus (cat-fish), Rana rugulosa (frog), Rana limnocharis* (small frog), Anas platyrhynchus domestica (domestic duck), Gallus gallus domesticus* (domestic chicken), Tupaia glis* (tree shrew) and Rattus rattus* (rattus rat or roof rat) were also found naturally infected with the third-stage larvae in the previous year and this year (*negative larvae this year). The larvae were found renetrate the stomach walls of the experimented mammals within a few minutes to a few days after experimental feeding. However subsequently some of these larvae could be found in visceral and other organs including heart blood, heart muscles, lung, trachea, intercostal and other muscles, and subcutaneous tissue as early as about one hour after the feeding experiment. Thereafter they were commonly seen singly in the flesh with early formation of a thin fibrotic wall around each living larva about 30 days after the infection. This wall was seen gradually to increase in thickness. If the host was sacrificed at much longer period after the nfection especially in one infected chicken the fibrotic cyst well around the healthy living larva was found 0.2 mm. thick in its leg's muscles when the animal was sacrificed 548 days after the experiment (Figure 1). The preliminary findings of such an experiment on certain animals was reported in the last year Annual Progress Report. The third-stage larvae were found in the livers of experimentally infected animals as long as 30 days after the feeding. In the present experiment larvae were fed to white mice. Upon sacrafice larvae were found encysted in the livers within 5 to 154 days. It may be assumed from this result that encysted larvae may also persist in human livers for many months.

Further experimental feeding were carried on to determine if species of animal other than fresh-water fish act as second intermediate hosts when feed on cyclops harboring second-stage or fully developed larvae of the worm and to confirm the previous findings of the same experiment of which the results are as follows:

Table IV Experimental infection of certain animals with third-stage larvae of G. spinigerum.

Species of animal	No.	Source and Number of infective larvae			Days after feeding	No. larvae positive			Remarks
		Fish	Snake	others		in liver	in muscle	in others	
Pisces (Fresh-water fishes)									
Ophicephalus striatus (Snake-headed fish)	3	—	40	6-small frog	1-11	3	2	0	2 fish positive
Clarias macrocephalus (Catfish)	8	5	—	32-chickens, frog, toad, rat	3-15	2	15	0	6 fish positive
Total	11	5	40	38	1-15	5	17	0	
Amphibia (Amphibians)									
Bufo melanostictus (Toad)	2	—	—	14-turtle, tree shrew, white mouse	7-20	0	3	4-stomach wall	
Rana rugulosa (Frog)	5	6	—	35-duck, rats, white mouse	5-26	0	11	2-stomach wall	
Rana limnocharis limnocharis (Small frog)	4	—	10	6-lizard	7	0	12	0	
Total	11	6	10	55	5-26	0	26	6	
Reptilia (Reptiles)									
Leiolepis belliana belliana (Ground lizard)	1	—	—	3-small frog	7	0	1	0	
Calotes versicolor (Tree lizard)	1	—	—	3-small frog	7	0	1	0	
Physignathus cocincinus (Agamid lizard)	3	—	—	16-monkeys, white mouse	7-25	0	7	2-stomach wall	

Species of animal	No.	Source and Number of infective larvae			Days after feeding	No. larvae positive			Remarks
		Fish	Snake	others		in liver	in muscle	in others	
Domonia subtrijuga (Turtle)	1	—	—	22-white mouse, rat	30	0	4	0	
Total	6	0	0	44	7-30	0	13	2	
Aves (Birds)									
Passer montanus (Tree sparrow)	1	—	—	9-white mouse	3	0	0	0	to be repeated.
Ploceus philippinus (Weaver bird)	2	—	—	11-white mice	9-13	0	2	0	1 bird positive.
Coturnix coturnix (Quail)	4	—	—	35-white mice, rats	7-31	0	7	0	2 birds positive
Anas platyrhynchus domestica (Domestic duck)	6	41		16-chicken, frog, white mouse	8-17	1	7	0	
Gallus gallus domesticus (Domestic chicken)	3	—	41	11-frog, toad	14-67	0	9	0	2 chickens positive
Gallus gallus domesticus (Domestic chicken)	1	—	—	—	—	0	0	0	Control chicken.
Gallus gallus domesticus (Domestic chicken)	13 (egg-laying)	251	723	1406-white mice, rats, quail lizards, small frog.	119-548	0	60	35-subcutaneous tissue	They were experimented for eggs examination. So far 796 eggs collected were found to be negative.

Species of animal	No.	Source and Number of infective larvae			Days after feeding	No. larvae positive			Remarks
		Fish	Snake	others		in liver	in muscle	in others	
Aves (Bird) continued.									During the period 7 chickens died 119-548 days after the experiment and showed 95 encysted G. spinigerum larvae.
Total	30	292	764	1488	3-548	1	95	35	
Mammalia (Mammals)									
Tupaia glis (Tree shrew)	2	—	—	11-frog, hamster	7-14	4	4	0	
Rattus rattus (Rattus rat)	7	17	154	3-duck	10 hours to 42days	31	45	36-lung, stomach heart, heart blood.	
Rattus exulans (Domestic rat)	5	22	23	43-white mice, rat, bird	13-116	1	36	0	
Mesocricetus auratus (Hamster)	34	—	6	145-rats, hamsters	5-156	0	98	1-stomach	
Rattus norvegicus var albinus (White rat)	71	—	278	914-white mice, rats, pigs, civet cat, turtle, lizard, frog.	5 mins. to 195 days	54	228	237-stomach, lung, diaphragm, esophagus, omentum, fatty tissue, trachea, heart, mesentery, kidney, blood.	

Species of animal	No.	Source and Number of infective larvae			Days after feeding	No. larvae positive			Remarks
		Fish	Snake	others		in liver	in muscle	in others	
Mus musculus musculus (White mouse)	161	95	1545	1069-white mice, rats, monkeys, rabbits, chicken, quail, guinea pig, tree shrew, lizards frogs.	15 hours to 155 days	778	1086	132-intestine mesentery, stomach omentum, fatty tissue, subcutaneous tissue, lung, pancreas, diaphragm.	
Mus musculus musculus (Pregnant white mouse)	28	44	150	129-white mice, rats, tree shrew, rabbit	5–19	99	122	13-intestine, diaphragm, stomach skin, kidney uterus, babies	Pregnant white mice, 27 mothers were positive. Of 208 babies examined two were positive with two larvae in costal region (un-encysted)
Veverricula indica (Civet cat)	1	8	50	–	287	0	71	1 adult male G. spinigerum from stomach. 1 larvae 1 male and 2 females (immature) from mesentery.	
Paradoxurus hermaphroditus canus (Palm civet cat)	1	–	30	–	241	0	8	0	
Macace irus (Crab-eating monkey)	2	–	–	23-white mice, chicken	3	2	0	1-esophagus	1 monkey positive.
Total	312	186	2236	2337	5 mins. to 287 days	969	1698	424	
Grand total	370	489	3050	3962	5 mins. to 548 days.	975	1839	467	

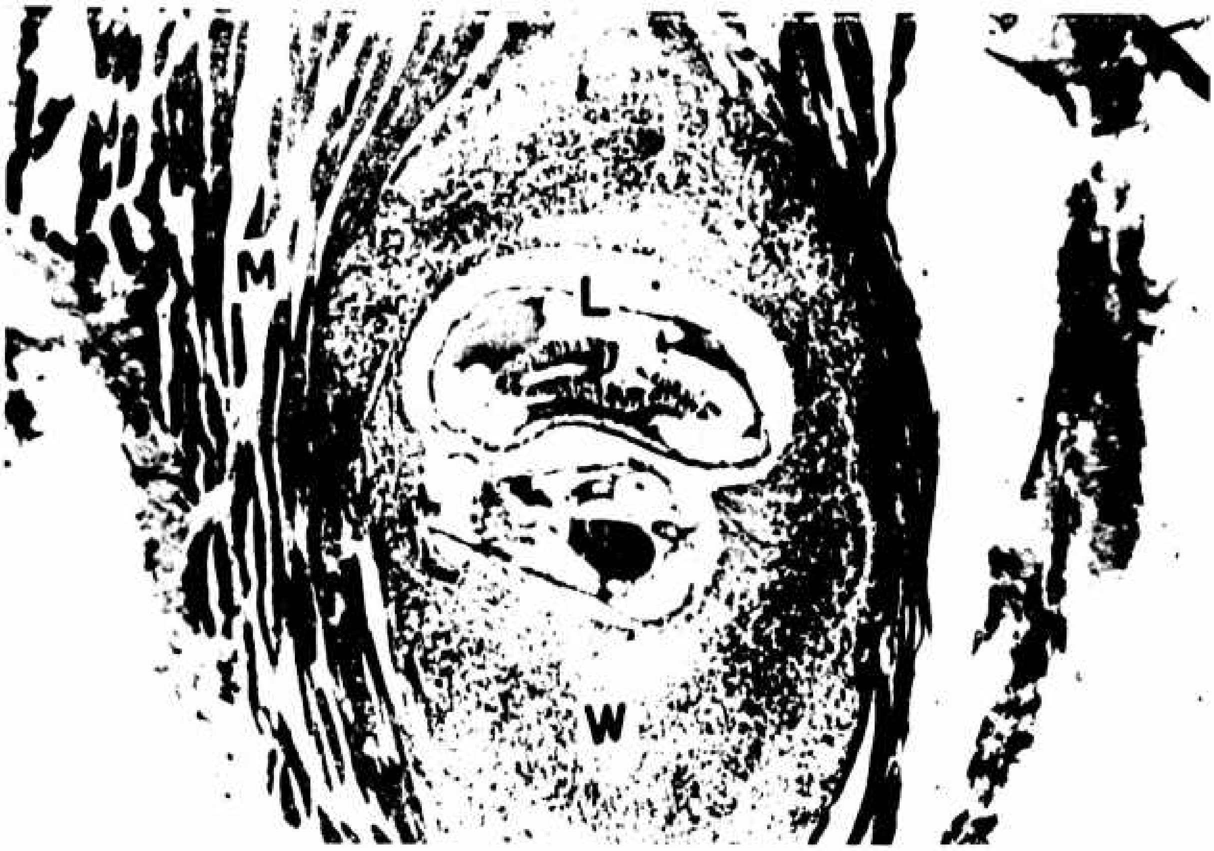

Figure 1. A microphotograph of the leg's muscles of a chicken sacrificed 548 days after being fed with G. spinigerum third-stage larvae shows an encysted living third-stage larva being surrounded with fibrotic cyst wall of about 0.2 mm. in thickness. (L larva, M muscle, W cyst wall.)

Table V Experimental infection of certain animals with G. spinigerum second-stage larvae (fully developed larvae in cyclops).

Species of animal	No.	No of larvae	No. of inf. cyclops	Days after feeding	No. larvae positive in livers	in muscles	in others	Remarks
Amphibia (Amphibians)								
Bufo melanostictus (Toad)	4	280	136	8-19	0	2	26-stomach, intestine	2 toads positive.
Rana rugulosa (Frog)	17	950	426	1-22	4	9	0	4 frogs positive.
Total	21	1230	562	1-22	4	11	26	
Reptilia (Reptiles)								
Boiga (Cat-eye snake)	1	75	42	17	0	1	0	
Varanus nebulosus (Monitor lizard)	1	100	56	21	0	1	0	
Physignathus cocincinus (Agamid lizard)	3	800	325	18-28	0	0	0	
Calotes versicolor (Tree lizard)	9	525	267	3-17	0	0	20-stomach, intestine.	3 lizards positive.
Total	14	1500	690	3-28	0	2	20	
Aves (Birds)								
Gallus gallus domesticus (Domestic chicken)	31	4977	2275	4-59	2	0	0	Only one chicken was positive 32 days after feeding.
Lonchura punctulata (Spotted munia)	2	60	31	7-9	0	0	0	to be repeated
Passer montanus (Tree sparrow)	2	62	25	3	0	0	0	to be repeated
Ploceus philippinus (Weaver bird)	5	231	133	3-5	0	0	0	to be repeated
Coturnix coturnix (Quail)	6	325	191	6-30	0	0	0	to be repeated
Total	46	5655	2655	3-59	2	0	0	

Species of animal	No.	No. of larvae	No. of inf. cyclops	Days after feeding	No. larvae positive			Remarks
					in livers	in muscles	in others	
Mammalia (Mammals)								
Tupaia glis (Tree shrew)	3	142	75	15-60	4	5	1-fatty tissue under external abdominal wall.	
Rattus rattus (Rattus rat)	3	158	76	15-49	2	12	1-subcutaneous tissue of abdominal wall	
Rattus exulans (Domestic rat)	2	150	56	17	9	1	0	
Mesocricetus auratus (Hamster)	1	76	37	90	0	6	0	
Sus scrofa domestica (Domestic pig)	7	1923	940	2-108	9	7	0	Three pigs were positive.
Rattus norvegicus var albinus (White rat)	2	118	63	90-180	0	11	0	
Mus musculus musculus (White mouse)	86	5904	2624	4 hours to 169 days	1028	704	99-intestine, stomach, lung, kidney, fatty tissue, subcutaneous tissue.	
Mus musculus musculus (Pregnant white mouse)	10	656	281	6-33	110	27	3-intestine, stomach, baby	9 mothers were positive. 78 babies were examined of which one of five unborn babies was positive with one third-stage larva in abdominal wall.
Total	114	9077	4119	4 hours to 180 days	1162	764	104	
Grand total	195	17462	8026	4 hours to 180 days	1168	777	150	

Of 21 amphibians 6 (29.0%) were positive for the third-stage larvae in gastro-intestinal tracts, livers and flesh from 1 to 22 days.

Of 14 reptiles 5(36.0%) were positive with the third-stage larvae in the muscles from 17-21 days and in their gastro-intestinal walls from 3-17 days. Of 46 avians only 1 two month-old chicken (2.2%) showed 2 third-stage larvae in its liver when sacrificed 32 days after feeding. Of 114 experimented mammals sacrificed from 4 hours to 180 days after feeding 110 (96.5%) (3 tree shrews, 3 rattus rats or roof rats, 2 domestic or polynesian rats, 1 hamster, 3 domestic pigs, 2 white rats and 96 white mice) showed the third-stage larvae in one or more organs namely liver, flesh, gastro intestinal tract, subcutaneous tissue, kidney and lung (Table V).

In this respect it is interesting to note that 3 of 7 experimented young domestic pigs were positive with 1 and 8 third-stage larvae in the livers of two pigs sacrified 33 and 45 days after feeding and the third pig showed 7 encysted third-stage larvae in the flesh 108 days after the experiment. The results of previous experimental feeding on 8 domestic pigs fed with the third-stage larvae showed positive larvae in livers and or flesh of 7 pigs sacrificed from 8 to 222 days after the experiment. Pigs therefore by these two experiments can be considered acting as paratenic and second intermediate host for the parasite. It is especially desirable to determine any natural infection of these animals in order to confirm the experiments because in many parts of Thailand, people eat fermented or pickled raw pork prepared in various forms of which the most popular one is called "Moo-nham" or in brief "Nham".

One of 5 fetuses of an infected pregnant white mouse was positive with one third-stage larvae in its abdominal wall 18 days after the pregnant mouse was fed on 100 second-stage larvae in 48 cyclops.

This present study has clearly proved for the first time that toad (Bufo melanostictus), frog (Rana rugulosa), cat-eye snake (Boiga), monitor lizard (Varanus nebulosus), tree lizard (Calotes versicolor) domestic chicken (Gallus gallus domesticus), rattus rat or roof rat (Rattus rattus), polynesian or domestic rat (Rattus exulans) and domestic pigs (Sus scrofa domestica) can serve as additional second intermediate hosts. It also confirmed the experimental findings reported last year that the hamster (Mesocricetus), white rat (Rattus norvegicus var albinus), and tree shrew (Tupaia glis) were proved to act as possible second intermediate host of the parasite as well as being suitable laboratory hosts for further studies on gnathostomiasis problems.

In summary more species of animals other than previously reported in the literatures have been added to the list of second intermediate and paratenic hosts by this year study as follows:

A. Additional species of animal proved to be second intermediate hosts after being fed on cyclops harboring second-stage larvae are toad (Bufo melanostictus), frog (Rana rugulosa), cat-eye snake (Boiga), monitor lizard (Varanus nebulosus), tree lizard (Calotes versicolor), domestic chicken (Gallus gallus domesticus), rattus rat or roof rat (Rattus rattus), domestic rat or polynesian rat (Rattus exulans) domestic pig (Sus scrofa domestica).

B. Additional species of animal proved to be paratenic hosts of the parasite in which the third-stage larvae of the worm could be transmitted by oral feeding and survived in livers and later in the flesh of animals without showing any morphological changes except a slight increase in size and reddish in color are the snake-headed fish (Ophicephalus striatus), cat-fish (Clarias macrocephalus), frog (Rana rugulosa), agamid lizard (Physignathus cocincinus), weaver bird (Ploceus philippinus), quail (Coturnix coturnix) roof rat rattus rat (Rattus rattus), polynesian rat or domestic rat (Rattus exulans) and palm civet cat (Paradoxurus hermaphroditus canus).

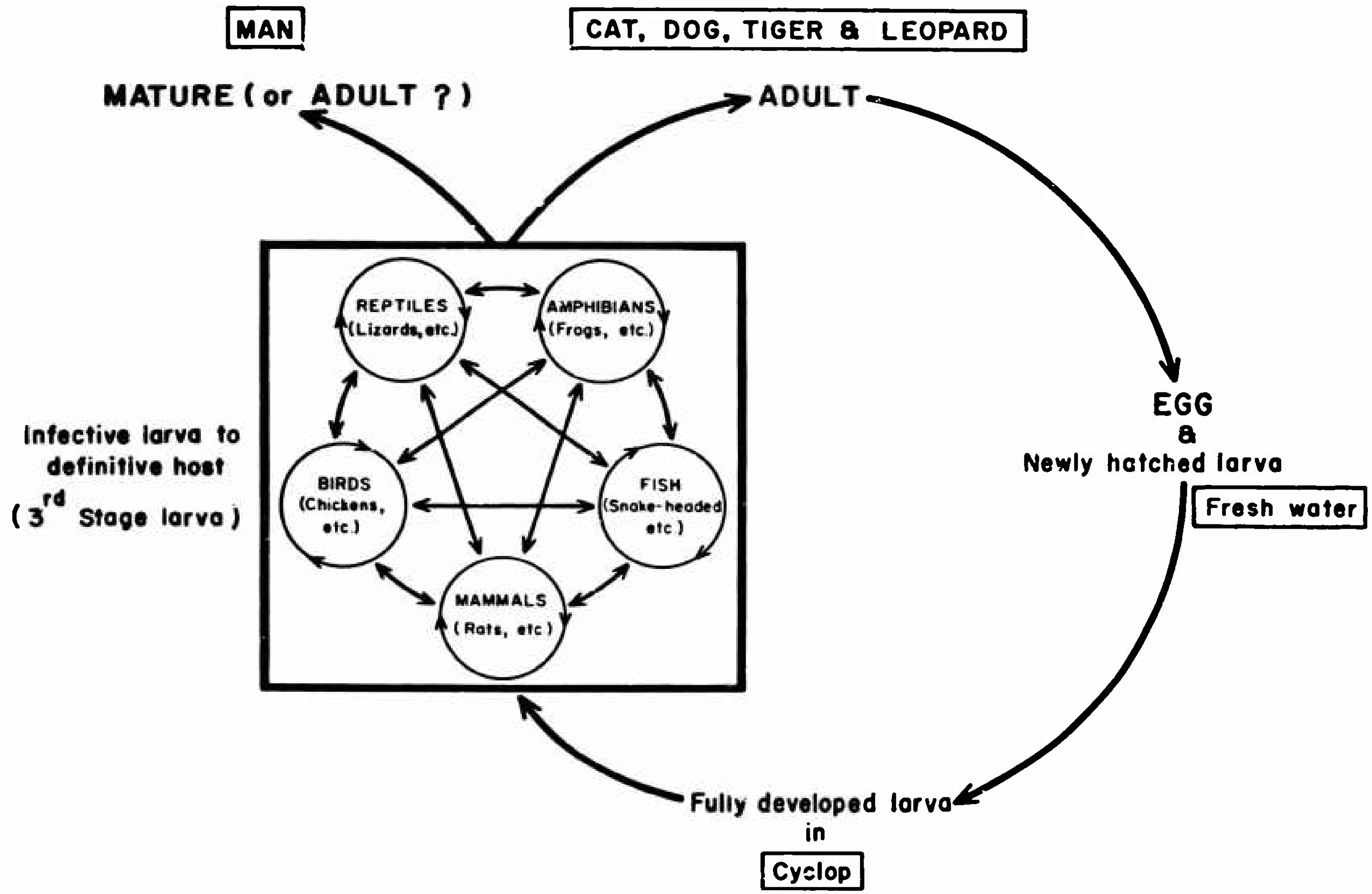

FIGURE 2. A DIAGRAM OF LIFE CYCLE OF GNATHOSTOMA SPINIGERUM IN THAILAND

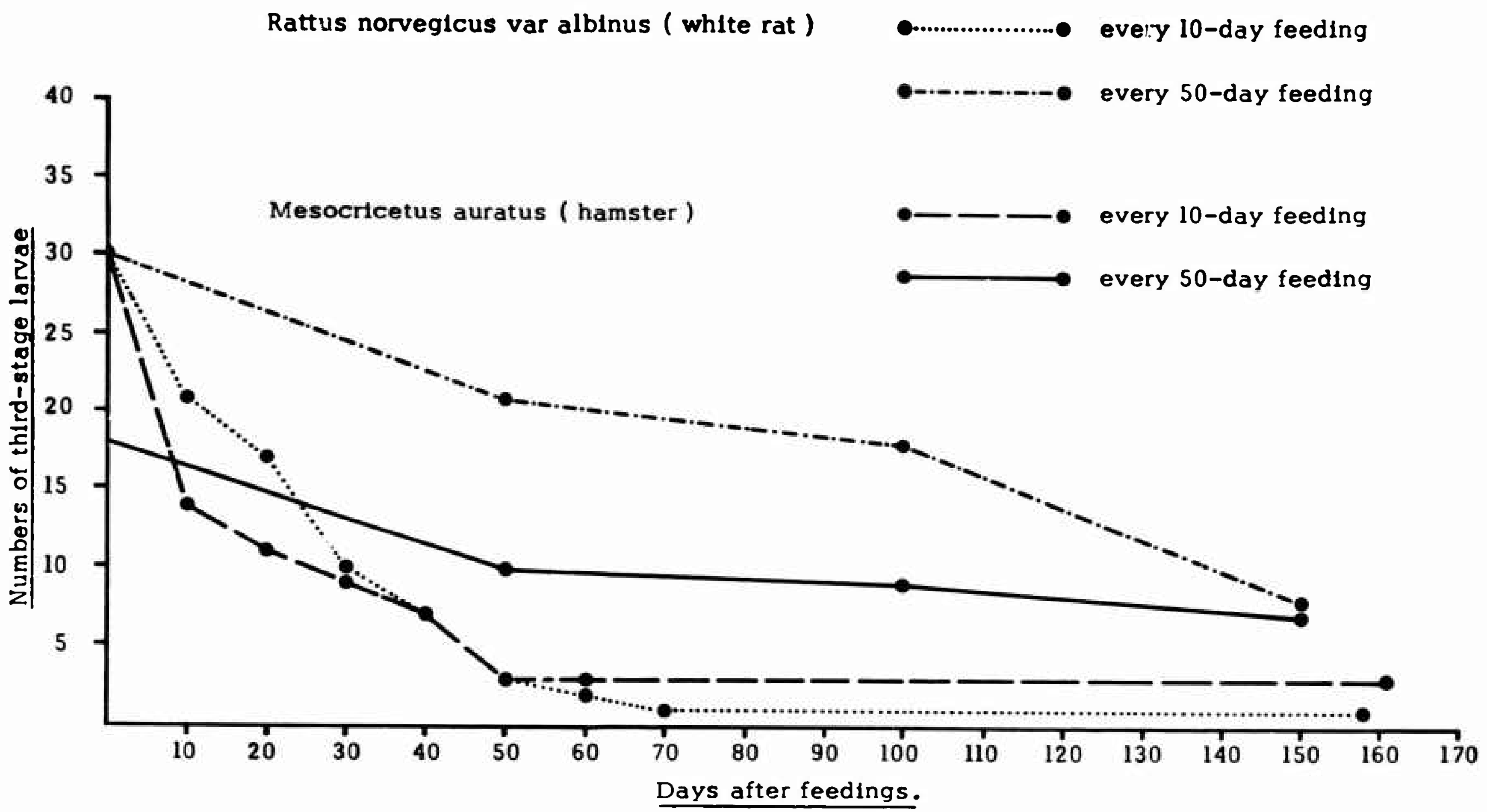

Figure- **3** Numbers of G. spinigerum third-stage larvae found in each group of 3 Rattus norvegicus var albinus (white rat) and Mesocricetus auratus (hamster) (the hamsters fed under 50-day plan were grouped in 2) sacrificed after being fed with the original 30 and 18 larvae and followed by successive feedings with the larvae obtained from preceding groups.

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The life cycle of G. spinigerum therefore now needs to be revised because of the additional discoveries and can be briefly shown in figure 2.

A new study was initiated to determine possibility of prenatal transmission in the experimental host. Twenty-eight pregnant white mice obtained from the Department Veterinary Medicine were each fed with 2 to 6 third-stage larvae. Some of the animals were given infective feeds every 2 to 6 days until the birth of the young. Two days after the birth of offspring, mothers and youngs were sacrificed and examined for the presence of the larvae. Of 208 suckling mice born from 27 infected mothers 2 were positive of which each had one unencysted third-stage larvae in costal muscles. The third mother had a larvae in the uterine muscles. This experiment therefore has clearly proved for the first time that prenatal transmission of larvae from mothers to offspring can occur in mice. Similarly, human gnathostomiasis may possibly be prenatally transmitted through infected mothers. The study on determination of the possible migration of the third-stage larvae into chicken eggs was continued with full co-operation of Kasetsatra University in providing on request free of charge the egg-laying hens and the feed for which we rae thankful. If these experiments prove to be positive, the infected hen's egg may constitute another important source of human gnathostomiasis. Each hen was repeatedly fed with about 5 third-stage larvae at intervals of two to three weeks. One hen was served as control. During the period covered by this report there were 555 eggs and 241 eggs for the last year all were found negative but more eggs need to be studied.

Another experiment was designed to determine whether the paratenic hosts may limit the number of the larvae infected after successive feedings of the parasite. For this purpose, groups of three Rattus norvegicus var albinus and groups of two to three Mesocricetus (hamster) were used. In one experiment the initial 30 third-stage larvae were fed to 3 white rats of each plan and only under the 10-day plan the initial 30 larvae were given to 3 hamsters while initial 18 larvae were fed to 2 hamsters under 50-day plan. Every 10 days and 50 days the animals were sacrificed and the larvae found from the first groups of experimented animals under the two plans (10-day and 50-day) were given orally to the second groups and subsequently all the larvae discovered from the second groups were fed to the third groups until both plans of feeding and sacrifice could be completed 150-160 days after the first infection. This experiment shows that there was a significant reduction of larvae after successive feedings and that this was more marked in the group given were fed to the third groups until both plans of feeding and sacrifice could be completed 150-160 days after the first infection. This experiment shows that there was a significant reduction of larvae after successive feedings and that this was more marked in the group given larvae under 10-day plan as compared to the group infected under 50-day program (Figure 3).

A further study on then number of eggs produced per day per female G. spinigerum was continued from the previous year on 8 more experimentally infected cats. The results showed each female worm in each cat produced daily the following numbers of eggs: 14,000, 18,000, 39,000, 45,000, 48,000, 64,000, 65,000. Tentatively the results of eggs-count made on 8 experimental cats for the present showed much variation from 14,000-65,000 eggs, however eggs production in 4 cats varied slightly from 39,000 to 48,000 eggs per day per female worm. During this period a naturally infected cat showed 95,000 eggs per day per female worm. In 3 experimentally infected dogs the eggs-count were about 42,000, 90,000, 75,000 per day per female worm.

The infectivity rate of the third-stage larvae fed to 8 cats and 3 dogs showed the following variations for cats 12+, 27+, 97+, 52+, 80+, 40+, and 13+ and in 3 dogs showed 1.8+, 11.8+, 43.+. The patent period in 6 experimentally infected cats were varied from 65 to 223 days.

With regard to the spontaneous cure of the infection with the worm in infected dogs and cats, it was shown that 5 experimentally and 1 naturally infected cats killed on 18 to 64 days after stools negative for gnathostome ova showed healthy stomachs and on worm in 3 cats killed on 40, 57 and 64 days but in two cats there was still in each stomach a small thickend area at the cardiac part when sacrificed 29 and 49 days after negative stool examinations. No worms were found in these old lesions. Only one cat killed after 18 days of negative stools showed one immature female in a small thickened area at cardiac part of the stomach. It is obviously proved by this preliminary experiment in cats that spontaneous cure of infection with G. spinigerum is possible after patent periods of 65 to 223 days. Further study on this problem is still in progress.

The effect of infection with G. spinigerum third-stage larvae on changes of peripheral white blood cells was again studied in 8 experimentally infected monkeys (Macaca speciosa) and Macaca irus and one control Macaca speciosa (#5) with the following results.

Monkey #1 showed slight increase of white blood cells and also an increase in eosinophilic cells after being experimentally fed 25 third-stage larvae obtained from a mouse. A few days later the animal was sacrificed and found to be infected with 11 encysted larvae of the worm in its flesh.

Monkey #7 fed with 500 fully developed larvae in cyclops also showed a slight increase of WBC from 43 days up to 53 days and a moderate increase in number of eosinophilic cells from 36 days up to 53 days, three days later it was killed and found to be infected with 3 encysted and 1 un-encysted third-stage larvae in its flesh and liver respectively.

Monkey #8 fed with 85 third-stage larvae obtained from a snake showed also a moderate increase of white blood cells from 15 days to 49 days but a great increase in number of eosinophilic cells from 15 days to 63 days and it showed 11 encysted third-stage larvae in the flesh when sacrificed 2 days after the last examination.

Monkey #10 fed with 85 third-stage larvae removed from a snake showed only a slight increase of WBC from 15 days to 21 days after feeding but a great increase in number of eosinophilic cells from 15 days to 3+ days. This animal is to be further studied.

Monkey #6 fed with 537 second-stage larvae in cyclops showed no increase in WBC or eosinophilic cells when examined from 48 days up to 65 days after the feeding. This monkey showed 2 encysted third-stage larvae of G. spinigerum in its flesh when it was sacrificed on 68 days after the infection.

Monkey #4 was given intraspinally 2 third-stage larvae obtained from a snake. It showed neither changes in total white blood cells nor eosinophilic cells when examined at intervals up to 528 days. It showed no infection on autopsy.

The other monkeys (#5, #9, and #13) are to be further studied.

The plan for preliminary determination of skin sensitivity of some laboratory animals experimentally infected with the third-stage larvae by intradermal testing with unfractionated lyophilized antigens prepared from adults and the third-stage larvae of G. spinigerum was further developed as described in the previous Annual Progress Report for 1 April 1965 to March 1966. During this period 8 monkeys (Macaca speciosa #1, #4, #6 and#7, Macaca irus #8, #9, #10 and #13) were fed with known numbers of G. spinigerum larvae and tested at interval with the antigens. The results of the experiment showed Monkey #1 (Macaca speciosa) gave a positive skin test from 3 days up to 51 days of the feeding. This animal showed 11 encysted third-stage larvae in its flesh when killed.

Monkey #6, #7 fed with 537 and 500 fully developed larvae in cyclops respectively and monkey #6 was skin tested positive on 48-65 days with 2 encysted third-stage larvae in its flesh on autopsy and monkey #7 was skin tested positive 36-53 days with 3 encysted larvae in the flesh and 1 unencysted larva in its liver.

Monkey #4 was skin tested positive on 356 days up to 531 days after intraspinal inoculation with 2 larvae obtained from a snake. However, the animal showed no lesions and no worm when sacrified on 567 days of the experiment.

Monkey #8 was fed with 85 third-stage larvae and an intraspinal inoculation of 5 larvae obtained from snakes showed negative skin test up to 365 days after intraspinal inoculation and 64 days after feeding.

Monkey ≠9 was intraspinally inoculated with 4 larvae obtained from a rat showed positive skin test on 153 days up to 293 days of the experiment. This animal is to be further studied.

Monkey ≠10 was fed with 85 larvae obtained from a snake showed positive for a short period when skin tested on 105 days of the experiment. It is also to be further studied. Monkey ≠13 was fed with 200 fully developed larvae in cyclops showed positive skin test on 21-96 days after feeding. The monkey is to be further investigated.

In summary on the result of skin test on monkey showed for 8 animals, 4 (≠1, ≠6, ≠7, and ≠13) positive skin reaction appear about 3 week up to 96 days of the feeding with third-stage and second-stage larvae. 2 monkeys (≠4 and ≠19) were skin test positive about 150 days up to 531 days after intraspinal inoculation wtih 2 and 4 third-stage larvae but one monkey (≠4) showed no lesion and no worm when sacrificed few days after the last test. 2 smonkeys (≠8 and ≠10) were both given intraspinal inoculation followed by oral feeding with the third-stage larvae obtained from snakes showed negative skin test in one monkey (≠8). This negative monkey had infection with 11 encysted third-stage larvae in its flesh when sacrificed about 65 days after feeding or 456 days after intraspinal inoculation. The positive monkey (≠10) is still kept for further study.

The results of skin test made on 6 white rats (Rattus norvegicus var albinus) and 4 rabbits (Oryctolagus cuniculus L.) are as follows:

Of 6 white rats 3 showed positive on 55 days up to 92 days after each being fed with 20 third-stage larvae and 3 others were negative when tested up to 146 days in 2 rats and 150 days in one rat after the experimental feeding each with 20 third-stage larvae. All rats were proved to be infected with 3 to 13 encysted third-stage larvae in their flesh on autopsy.

Of 4 rabbits, 3 were skin test positive on 71 to 104 days after being fed with 4, 20, 20 third-stage larvae respectively. The negative rabbit was fed with 20 larvae and died 43 days after the experiment. All rabbits were proved to be infected 2 to 14 encysted third-stage larvae on autopsy.

Pathological study

A detailed study to determine pathological changes of organs in white mouse (Mus musculus musculus) after being experimentally fed with 5-8 third-stage larvae and sacrificed on 1 day up to 6 months after the feeding was undertaken of which the preliminary results of 1 day up to 12 days are summarised as follows: Of 6 white mice sacrificed 1-3 days, one stomach showed 2 larvae in its submucous and muscular layers which had congestion of blood vessels, early pressure atrophy of muscles around the larvae with few lymphocytic neutrophic polymorphonuclear leucocyte infiltration. The small intestine showed at few places of similar reaction to that of the infected stomach with the presence of the larvae. Localized peritonitis was found around the infected areas of stomach and intestine and mainly infiltrated with lymphocytes, some polymorphonuclear leucocytes and few eosinophilic cells. All 6 livers showed a few congested and hemorrhagic areas together with a few yellowish white tracts of about 1.0 to 2.0 mm. X 0.5 to 1.0 mm. in size which were first seen at the lower surface and followed later at the upper surface of the left lobe. On the lower surface of left lobe of some livers there were little grayish white exudates similar to that found around the infected stomach and intestine. One to 4 third-stage larvae were found in only 3 of 6 livers examined. Microscopically, sections of the liver revealed cystic spaces of varying size. The cyst is composed of fibrinoid material as a wall and contain lymphocytes, neutrophilic polymorphonuclear leucocytes and a few eosinophiles. Some of these cysts contain purulent exudates consisting mainly of neutrophilic polymorphonuclear leucocytes and cell debris thus becoming abscess of varying size. The liver cells around the abscesses showed not much change except for some pyknotic nuclei. The blood vessels are severely congested and in few places there are small collections of red blood cells outside the vessels. In two mice there were a few small hemorrhagic areas scattered on the upper part of the costal surface of the left lungs and also of the right lung in one mouse.

Larvae were also found lying freely in costal muscles of 2 mice and in abdominal muscles and right hind leg muscles of one mouse with congestion of blood vessels around the larvae.

In the mesentery of 1 mouse a slight congestion of blood vessels was seen around the unencysted larvae.

Of 6 mice sacrificed 4-7 days after the feeding experiment each with 5-8 third-stage larvae, 3 showed 3 to 4 larvae in costal muscles and livers, 2 had 1 to 5 larvae in the livers and 1 had 1 larvae in its costal muscles. Pathologically the livers showed similar changes as seen in the group infected for 1-3 days except more damage was seen in the left lobes of livers and in one mouse (#123) the right lobe started to show few small areas of congestion and fibrinous changes at its left side adjacent to the left lobe otherwise normal.

Of 7 mice sacrificed 8-12 days after each being fed with 5 third-stage larvae, 4 showed 2 to 4 larvae in livers and muscles of neck, fore-leg, intercostal and abdominal wall and in mesentery, and 3 had 2-4 larvae in the muscles of fore-leg, abdominal wall and costal region.

On the upper and lower surfaces of left and right lobes of all livers there were many small irregular grayish white tracts of about 1.0 to 2.0 mm. X 0.5 to 1.5 mm. among which a few small hemorrhagic and congested spots were seen. Infected muscles and mesentery were slightly congested around the larvae other-wise normal. Microscopically the livers, muscles and mesentery showed similar pathological changes to those of 4-7 days after being infected except there were more areas of the liver tissue involved.

To help elucidate the epidemiological aspects of gnathostomiasis, an investigation for naturally infected cyclops with fully developed larvae (second-stage larvae) of G. spinigerum was undertaken by collecting and examining 5150 cyclops from few selected ponds of the South and from some ponds in Bangkok area. No infected cyclops were found.

Summary: Human gnathostomiasis in Thailand is highly endemic. The disease seems to be least endemic in the southern region, however it can be reasonably assumed as a result of personal visit and communication to four southern provinces that approximately 15 to 20 cases may be expected yearly. With regard to animal definitive hosts acting as reservoirs of the infection discovered during this year. 106 (2.3%) dogs in Bangkok and Thonburi except one in Nakornsrithammarat were positive only during the rainy and early part of dry seasons of the year. Other animals were negative.

To determine the natural infection of cyclops with gnathostome larvae 5,150 cyclops from Bangkok and the South were found to be negative.

Examination for natural infection of animals with G. spinigerum third-stage larvae showed two more species of animals than previously recorded namely Ophicephalus lucius (snake-headed fish) and Trimeresus gramineus (green pit viper), This year among animals found to be naturally infected with the larvae, snake-headed fish, eels and frogs still showed higher a prevalence of infection than other species.

Experimentally 23 species of animals in five classes were found to be acted a sparatenic hosts of which 9 were newly recorded. Of these 9 species, 4 including Physignatus cocincinus (weaver bird), Coturnix coturnix (quail) and Rattus exulans (polynesian rat) were not as yet found naturally infected with the larvae 9 species of animal also were newly proved to be infected with the third-stage larvae after being fed with. fully developed larvae in cyclops (second intermediate hosts).

Additionally some of these experimentally infected animals were common laboratory amimals and can be used effectively as experimental hosts.

An experimental study to determine prenatal transmission of the larvae in pregnant white mice has definitely shown that 2 suckling mice from two pregnant white mice were positive, each with one unencysted third-stage larvae in its costal muscles.

The present findings of new natural and experimental second intermediate and paratenic hosts are expected to further contribute to the existing knowledge on life cycle and some epidemiological aspects of G. spinigerum. For clarification of the above-mentioned knowledge gained by this study, a revised diagram of life cycle of the helminth is also presented.

The investigation on egg production and spontoneous disappearance of adult worms from infected cats being continued from last year and egg production of the worms in infected dogs also initiated this year has given further knowledge on the spread of infection. Infectivity rates of the third-stage larvae in cats and dogs showed much variations from 13.0% to 97% in cats and 1.8% to 43% in dogs.

The preliminary result of the study on detailed pathological changes of the infected organs of experimented mice were presented from 1 up to 12 days of infection. A plan of study on the development of fully developed larvae in cyclops after being fed to mice and pathological changes of the infected organs caused by their presence on different days was also initiated of which the result will be presented in the future. These experiments are still in progress.

Results of preliminary studies of skin sensitivity on experimentally infected laboratory animals including 6 white rats, 4 rabbits and 8 monkeys were presented. This study is to be continued.

The result of a study made on white blood cell changes in peripheral blood of 8 monkeys after fed or and inoculated with the third-stage larvae obtained from second intermediate hosts or after being fed with second-stage larvae in cyclops it has been shown that some infected monkeys had a short period of leucocytosis and eosinophilia. Further study on this problem is to be undertaken before any conclusion is made.

Publication: Further Investigation on Natural and Experimental Hosts of Larvae of Gnathostoma spinigerum in Thailand by Svasti Daengsvang, Praja Thienprasitthi and Pasoog Chomcherngpat. Amer. J. Trop. Med. and Hyg. 15: 727-729.

SEATO Clinical Research Study on Growth and Development

Coordinator: Aree Valyasevi, M.D.	Chief, Thai Component Clinical Research Center
Principal Investigator:	Major Chitti Palavatana, MC, RTAH
Associate Investigators:	Chaiyan Kampanart-Sanyakorn, M.D. SGT John T. Lebow
Period of Report:	1 April 1966-31 March 1967

GENERAL INFORMATION

To our knowledge basic data regarding Growth and Development of normal Thai subjects have not been documented. In view of genetic, nutritional and environmental differences between Eastern and Western cultures the standards for growth and development of Western peoples can not be arbitrarily transposed to Thai subjects. It is also necessary to obtain such basic information for understanding and interpretation of the effects of various metabolic and nutritional disturbances on growth and development.

STUDY REPORT

1. Title: Radiologic Study of the Development of Ossification Centers in the Extremities and Bone Age in Thais.

Principal Investigator:	Major Chitti Palavatana, MC, RTAH
Associate Investigators:	Chaiyan Kampanart-Sanyakorn, M.D. SGT John T. Lebow

Object

To determine bone age standards for normal Thais.

Progress

Subjects are obtained from the well-baby clinics of Children's Hospital, Pra Mongkutklao Hospital and from selected schools in Bangkok. The criteria for selection of subjects and the method of study have been as previously reported.

Thus far, 586 subjects have been studied. Not all of the films have been read, but it would appear that bone there is no significant difference from American standards. The results will be compiled and analysed statistically.

SEATO MEDICAL RESEARCH STUDY ON HEMATOLOGY

Coordinator:	Dale R. Snyder, LTC, MC, Ch. Lab. Svc.
Principal Investigators:	Chulee Mitrakul, M.D., D.Sc. Natth Bhamarapravati, M.D. Prasertsri Sitachitt, Ph.D. Ray A. Olsson, LTC, MC
Associate Investigators:	Supa Na-Nakorn, M.D. Sudsakorn Tuchinda, M.D. Sompote Bukkhavesa, M.D. Prawase Wasi, M.D.
Assistant Investigators:	Surin Charoensiri, B.Sc. Marasri Lamyaithong, B.Sc.
Period of Report:	1 April 1966 31 March 1967

STUDY REPORTS

1. Title: Pathology of Abnormal Hemoglobin Diseases Seen in Thailand

Principal Investigator: Natth Bhamarapravati, M.D.

Associate Investigators: Supa Na-Nakorn, M.D.
Sudsakorn Tuchinda, M.D.
Sompote Bukkhavesa, M.D.
Prawase Wasi. M.D.

Period of Report: 1 April 1966-31 March 1967

Objective:

The objective of this study is to evaluate the morphological changes in the various organs of Thai patients who carry genes for abnormal hemoglobins. These genes may occur in combinations and produce various forms of diseases. The common forms with severe clinical pictures are beta thalassemia hemoglobin E disease, hemoglobin H disease, and hydrops fetalis due to Bart hemoglobinopathy. Other individuals may harbor these genes in heterozygous form with mild or inapparent clinical manifestations. Of interest would be the evaluation of host responses of these subjects to some of the undesirable environmental factors e.g. infectious diseases or malnutritional factors. Base line information is needed, however, before further study can be made. Studies have been planned in stages as follows:

1. The pathology seen in fatal cases of beta thalassemia hemoglobin E disease. This part of the study is completed, and the final paper will be published in June 1967.

2. The pathology of hemoglobin H disease. This form of abnormal hemoglobinopathy is fairly common in Thailand (Minnich et al 1956, Na-Nakorn et al 1965). Hemoglobin H disease is generally considered to occur as a result of defective alpha-chain synthesis from two alpha-thalassemia genes, leading to more production of beta-chains. The clinical manifestations of hemoglobin H disease are generally considered to be milder than thalassemia hemoglobin E or thalassemia major (Na-Nakorn et al 1965). Seven autopsy cases of hemoglobin H diseases have been collected and reviewed (See Table I). At autopsy, jaundice was noted in 5 cases. Some degree of retardation of physical growth was present in three cases. Mongoloid facies, i.e. flat and broad face with prominent cheek bone and sunken nose, was seen in the 10-year-old male but not in others. Three cases had splenectomy for a period of 3 months to 2 years, while in one case the patient died because of bronchopneumonia in the post operative period. Six cases showed definite cardiac hypertrophy based on weight and measurement of cardiac walls. Iron containing pigment is not present in the myocardial fibers, but in two cases hemosiderin pigment is noted in the endocardial and pericardial tissue. Perinuclear lipochrome pigment is increased in myocardial fibers in 4 cases.

In the pancreas, only 2 cases show a mild deposit of hemosidering granules in the acinar cells. None of the adrenal glands show iron deposit. In one case the skin reveals heavy deposit of hemosiderin in the connective tissue cells of the corium and in the subcutaneous fat. The livers of these patients were considered to be enlarged, considering the normal weights of each age group. A moderate amount of hemosiderin (3+) is present in the liver cells, whereas in the Kupffer cells a relatively small amount of iron containing pigment is present. Some enlargement of the portal areas is seen, mainly due to an increase in

connective tissue and proliferation of perilobular ductules but there is no definite formation of connective tissue septa, and regenerating pseudolobules are not seen. The Kupffer cells were markedly hypertrophic and showed excessive erythrocytic phagocytosis, especially in cases where splenectomy had been done for some time. Extramedullary hemopoiesis was noted in two cases and in one of these a large erythroblast was seen. Cholestasis was not observed in any case. In one case, submassive hepatic necrosis was seen which was considered to be fulminating viral hepatitis. The spleens were enlarged in all the cases. In two cases siderotic nodules consisting of iron containing crystals surrounded by collagenised fibrous tissue are seen in the trabeculae of the spleen. Trapping of erythrocytes in the Bilroth cords was prominent in all cases. The amount of lymphoid tissue in the white pulps was slightly reduced in one while in others it was within normal limits. Germinal center reaction is not noted. Focal extramedullary hemopoiesis was seen in the sinusoids in one case. Fibrosis of either red pulps or white pulps was not present. The kidneys of these patients showed a mild to moderate degree of glomerular enlargment. In two cases focal endothelial cell proliferation was observed. Hemosiderin pigment was present in the straight tubules in five of six cases. A small number of bile casts and hemoglobin droplets was also observed. The bone marrow revealed marked depletion of both erythroid and myeloid elements in one case with proliferation of macrophages showing active phagocytosis of cellular debris. Other cases showed hypercellularity of bone marrow of both erythroid and myeloid elements. In one case, plasma cells were markedly increased in number.

Comparing the findings observed in this series of hemoglobin H disease with those seen in beta-thalassemia hemoglobin E disease, a few observations can be made. Subjects who harboured hemoglobin H (hemoglobin A and H) do show pictures of chronic hemolytic anemia, but the morphological changes appear to be less severe than in the other condition. Most of the cases of hemoglobin H diseases survive to adulthood or show manifestation of the disease later in life. In our autopsy experience, during 1960-1966, only two autopsy cases of hemoglobin H disease in children were encountered out of a total of seven cases of this type, while fourteen out of twenty autopsy cases of beta thalassemia hemoglobin E disease were in pediatric age group. The extent of hemosiderosis is much less in the spleen and other visceral organs in hemoglobin H disease but hemosiderosis of the liver is somewhat comparable to what was observed in thalassemia hemoglobin E disease. The degree of extramedullary hemopoiesis was relatively mild. The spleens of thalassemia hemoglobin H disease appear to be effectively trapping the erythrocytes since the Bilroth cords are bulging and degenerating erythrocytes are present. The liver changes in hemoglobin H disease are also relatively milder. No formation of pseudolobules nor extensive connective tissue septum formation is noted.

3. The pathology of hydrops fetalis associated with hemoglobin Bart's. Hydrops fetalis associated with high production of hemoglobin Bart's was first recognized by Lie In Jo in Malaysia in 1962. Subsequently, it was shown that in this condition there is an inherited hemozygosity of alpha-thalassemia genes resulting in a suppression of alpha chain synthesis and over production of gamma chain to form hemoglobin Bart's (hemoglobin gamma 4). The pathology of this interesting condition has only been briefly described. A series of 20 autopsy cases of hydrops fetalis by the Department of Pathology of Chiengmai Medical School has just been made available for a cooperative study on the pathology of this condition to be conducted jointly between Dr. Anong Nontasut and the principal investigators: The result of this study is not available.

Summary:

Pathological studies in fatal cases of hemoglobin H disease have been made. In general the findings resemble those in Beta thalassemia hemoglobin E disease but the morphological damages are relatively milder. The spleen in Beta thalassemia hemoglobin H patients appear to trap more effectively the abnormal erythrocytes. Liver disease does not progress as much as in Beta thalassemia hemoglobin E disease. Less hemosiderosis and extramedullary hemopoiesis are also observed.

Publications:

"Pathology of abnormal hemoglobin diseases in Thailand." American Journal of Clinical Pathology, June 1967.

TABLE I

CASE	AGE	SEX	DURATION OF SYMPTOMS	HEMOG' LOBIN LEVEL (a)	NO. OF BLOOD TRANS-FUSIONS (b)	DURATION AFTER SPLENEC-TOMY	CONDITIONS AT DEATH	DEVELOPMENT
1	2	F	1 mos.	3.3 gm%	Several	—	Acute non-specific ulcerative colitis	Retarded
2	10	M	3 yrs.	6.4 gm%	2	3 mos.	Acute hepatic failure	Retarded
3	21	F	10 yrs.	—	3	—	Hemolytic crisis	Normal
4	25	F	6 yrs.	6.9 gm%	Several	2 yrs.	Heart failure-post op. manual extraction of placental adhesions	Normal
5	29	M	29 yrs.	4.9 gm%	5	—	Congestive heart failure Pregnancy 28 weeks	Retarded
6	32	M	5 yrs.	4.2 gm%	5	4 yrs.	Tetanus-chronic ulcer of left leg	Normal
7	33	F	19 yrs.	3.9 gm%	11	3 days	Bronchopneumonia	Normal

Title: The Karyotypes of the Hemoglobinopathies and Glucose-6-Phosphate Dehydrogennase Deficiency in Thai.

Principal Investigator: Chulee Mitrakul, M.D.*

Period of Studied: July 1965 – December 1966

Background of Study:

It is known that the incidences of the hemoglobinopathies and the G-6-PD deficiency are quite high among the Thai population. Various investigations of these problems, including the genetic patterns, have been carried out.

Since the etiologies of these disorders are as yet undetermined, it is desirable to investigate as many aspects as possible.

Our purpose here is to evaluate the karyotypes of these two diseases.

Materials:

In our original plan, five groups of subjects will be submitted for chromosome studies:

1. One hundred control studies from normal, healthy individuals.
2. Twenty subjects who manifest definite G-6-PD deficiency, both clinically and by the assay method for the enzyme level.
3. Twenty subjects with Thalassemia major.
4. Twenty subjects with Hemoglobin E disease.
5. Twenty subjects with Hemoglobin H disease.

All of these subjects will be of various ages with no history of any significant irradiation, nor any detectable abnormalities or any other diseases at the time of the studies.

All the subjects will also have a complete blood count, hemoglobin electrophoresis and the G-6-PD determination performed at the time of the culture of the leukocytes.

Methods:

1. Chromosome study:

Peripheral white blood cells or bone marrow was used as indicated, in order to obtain the best results.

Techniques of R.G. Schertz et al and K.A. Kiosssoglou were selected for peripheral blood and bone marrow respectively.

* Dip. of American Board of Pediatrics; D.Sc. (Penn).
Pediatric hematologist, Chulalongkorn Hospital

a. Peripheral blood: Eight to ten ml. of heparinized peripheral blood was collected into a sterile culture tube. The tube allowed to stand in a refrigerator for 3-4 hrs. A few ml. of buffy coat were aspirated and poured another sterile tube containing: TC 199 media, phytohemagglutinin penicillin & streptomycin. This mixture was incubated at 37°C for 72 hrs. To stop the growing cells at metaphase, colchicine was added. The mixture was again incubated at 37°C for another 4 hrs. The tube was centrifuged at 800 r.p.m. for 10 min., and then the supernatant poured off. The hypotonic solution, 0.95% sodium citrate, was then added to the "button" of the cells. This mixture was incubated at 37°C for 10 min. Following incubation the tube was centrifuged and the precipitate saved. The sediment was washed with glacial acetic-methanol fixative for 3-4 times. Two-three drops of a suspension were then delivered on to the surface of a clean slide. The surface of the slide was ignited for a few seconds. The prepared slides were then dyed with Giemsa's stain, and Mounted in Permount. The preparations were now ready for the microscopic examination.

b. Bone marrow: The technique is more or less the same as used for the peripheral blood except the stage of culture in the TC 199 media was not needed.

2. Hemoglobin electrophoresis:

The starch gel method was selected.

3. The alkaline denaturation test:

Followed the method of Singer & Chernoff.

4. The G-6-PD determination:

a. Spot test using Biochemia kit or Matulsky's technique was used as available.

b. For the bioassay technique, C.F. Bochringer & Sochne's method was used.

Results:

As shown in tables 1,2, and 3 for the Hemoglobin E disorders, Hemoglobin H disease and Thalassemia major respectively.

The results, though, are not yet conclusive. For the majority of cells studied, 176 out of 234 cells (72.5%) in Hb E disorders and 114 out of 115 cells (73.5%) of the Hemoglobin H disease 46 chromosomes were observed.

Thirty nine of 46-chromosome containing cells in Hemoglobin E disorders were karyotyped, of these 4 cells show certain, but not constant abnormalities.

In hemoglobin H disease series, the number of karyotyped cells is still too small for any discussion. Though the incidences are less common in our hospital, quite a number of Thalassemia major and the G-6-PD deficient patients were submitted to the study. The results were, unfortunately, not satisfactory.

Since there may be chromosomal aberations of varying types associated with the hemoglobinopathies studied, a continuation of this study is planned. Interpretation of the significance of these observations must be deferred until studies of control preparation are completed.

SEATO MEDICAL RESEARCH STUDY ON LABORATORY ANIMALS

Coordinator: Jack S. Stanton, Major, VC, Chief, Department of Veterinary Medicine

Principal Investigators:	Paul C. Smith, CPT, VC Jack S. Stanton, MAJ, VC William E. Vick, CPT, VC
Associate Investigators:	Richard D. Buchanan, CPT, MC Francis C. Cadigan, Jr., MAJ, MC David M. Robinson, LTC, MC Robert L. Taylor, LTC, MSC
Assistant Investigators:	Verachart Chaicumpa, D.V.M. Kwanyuen Lawhaswasdi, D.V.M. Jack M. Preston, SP4 Prayot Tanticharoenyos, D.V.M. Lenly D. Wetherald, SSG

Period of Report: 1 April 1966 – 31 March 1967

STUDY REPORTS

1. Title: "Nutritional and Health Requirements for Development and Maintenance of Conventional Animal Colonies".

Principal Investigators:	Paul C. Smith, DPT, VC Jack S. Stanton, MAJ, VC William E. Vick, CPT, VC
Associate Investigators:	Richard D. Buchanan, CPT, MC Francis C. Cadigan, Jr., MAJ. MC' David M. Robinson, LTC, MC Robert L. Taylor, LTC, MSC
Assistant Investigators:	Verochart Chaicumpa, D.V.M. Kwanyuen Lawhaswasdi, D.V.M. Jack M. Preston, SP4 Prayot Tanticharoenyos, D.V.M. Lenly D. Wetherald, SSG E6

Study Reports: Nutritional and Health Requirements for Development and Maintenance of Conventional Animal Colonies" -- page 2

Objective: The objective of this study is to produce, procure, and maintain healthy laboratory animals to support investigative programs and to determine procedures and standards for production and maintenance of conventional laboratory animals under conditions existing in this area.

Description: Surveys have been instituted in all rodent production colonies to determine the incidence and extent of parasitic infestations, latent virus infections, bacterial infections and presence of pathological conditions which may affect the outcome of investigations utilizing these animals as biological models. Measures are taken to correct these conditions when feasible. Vigorous standards are maintained in all rodent colonies for selection of breeder stock in order to increase litter size, growth rates, weaning rates and to insure as much uniformity of animals as possible.

All animals purchased for investigative work are examined at the time of purchase and all subhuman primates are quarantined prior to issue to investigators. Individual clinical records are maintained on all subhuman primates and necropsies are performed on all animals that die within colony.

Progress: A survey of the mouse colony revealed that 74.6% of the adult mice examined were infested with oxyurids (Syphacia obvelata and Aspicolaris tetraptera) and that 27.3% of the mice examined were harboring Hymenolepis citelli. A limited anthelminthic trial on adults from the colony indicated that piperazine citrate administered at a dosage of 400 mg per 100 cc of drinking water given to the animals for 7 days, followed by 7 days with untreated water and another 7 days with treated water greatly reduced or

eliminated the oxyurid infestation with no apparent ill effects to the mice. Such medication is easily accomplished and does not require extra handling of the mice. Treatment has now been started in the production colony and results to date are excellent. Feedbins located in the mouse building and containing locally procured feed for other animals were found to be contaminated with meal beetles. Such feedbins have been moved from the premises of the mouse building areas. With the elimination of these intermediate hosts and with the normal replacement of infested breeder stock, tapeworm infestations are greatly reduced.

No infestations of the tropical rat mite (Ornithonyssus bacoli) have been found during the period of this report.

Sera from 95 randomly selected adult mice were submitted for antibody determination against the following latent mouse viruses: Pneumonia Virus of Mice (PVM), Polyoma, K Virus, Mouse Adenovirus, Mouse Hepatitis Virus, Reovirus 3, Sendai, and GD VII. Results of these antibody determinations are summarized in Table I.

Table I

MURINE VIRUS ANTIBODY DETERMINATION

Virus	Serum Dilution				Total Positive	% Positive
	1:10	1:20	1:40	1:80		
Pneumonia Virus Mice	NT	0	0	0	0	0.00
Polyoma	NT	0	0	0	0	0.00
K Virus	0	0	0	0	0	0.00
Mouse Adenovirus	2	1	0	0	3	3.16
Mouse Hepatitis Virus	2	1	0	0	3	3.16
Reovirus 3	NT	14	3	3	20	21.53
Sendai	11	28	11	5	55	52.63
GD VII	NT	18	20	20	58	61.05

NT not tested

Total Number Sera Tested 95

Of 150 adult mice necropsied, 118 (78.6%) had no gross pathological lesions. Gross lesions most frequently found were consolidated lungs (12%) and discolored or mottled livers (11.3%). Microscopic examination of lung tissue showed pneumonitis and broncho-pneumonia. Most microscopic liver examinations were unremarkable. Specimens submitted for bacteriological examination revealed no bacteria considered to be pathogenic.

During September and October there was a reoccurrence of a disease which had been previously reported in the rat colony. The disease produced a high mortality (45%) in suckling rats and was clinically characterized by stunting, listlessness, dehydration cyanosis and death. The disease had previously been presumed to be of viral etiology, however cell free filterates of intestinal contents and 20% brain suspensions failed to infect mice by either intraperitioneal or intracranial inoculation. Ingesta, feces, and intestinal contents force fed to suckling mice also failed to produce disease in mice. Attempts to isolate a viral agent in monolayer cultures of primary hamster (HK) cells and in continuous line monkey kidney (MK) cells were unsuccessful. Bacteriological cultures were negative for pathogens. The only gross pathological lesion consistently found at necropsy on baby rats which had died was a gaseous distention of intestinal tract. No blockage of the intestinal tract was evident although minute particles of sawdust could be found in the lumen. 10 moribund baby rats were sacrificed and necropsied with the aid of a dissecting microscope. Blockage of the ilio-cecal orifice by particles of bile stained material was present in all animals. Microscopic examination showed the obstructions to be of ligneous nature. Locally purchased sawdust bedding (10% of which passed a 100 mesh screen) was suspected of being the cause of the deaths. Subsequently only sawdust bedding retained by a 20 mesh screen has been used and known suckling mortality has been reduced to less than 2% as compared with 45% when unscreened sawdust was used.

Animal production figures for the rodent colonies are shown in Table II.

Table II

ANIMALS BORN IN RODENT COLONIES

Animals	Number of Litters	Number of Animals	Average/Litter
MICE	28,864	265,121	9.18
RATS	1,232	10,520	8.53
HAMSTERS	1,764	12,115	6.86
GUINEA PIGS	455	1,506	3.31
Total	32,315	289,262	

Animals issued to investigators during the report period are shown in Table III.

Table III
ANIMALS ISSUED

	PRODUCED			PURCHASED		
	Suckling	Juvenile	Adult	Suckling	Juvenile	Adult
MICE	69,435	37,562	7,247			—
RATS	148	4,094	286			—
HAMSTERS		2,o25	276	—	—	—
GUINEA PIGS		676	208	—	700	—
RABBITS			—	1,799	—	682
GIBBONS		-	-	—	48	28
MONKEYS	1	—	—	—	—	72
CHICKENS						3

Total Animals Issued 125,213
Embryonated Eggs Issued 967 dozen.
Animal Blood Issued 36,000 cc.

During November one juvenile gibbon at Prabuddahbat died of cysticercosis. The cysticerci were identified as larval stages of Taenia solium. Efforts to trace the source of infection were unsuccessful. In an effort to determine if the gibbon could possibly harbor adult Taenia solium and if cysticercosis could result from antoinfection, 15 viable cysticerci obtained from pork at a local abattoir were fed to another juvenile gibbbon. On the 53rd day after feeding the cystecerci, Taenia eggs were recovered from the gibbon's stool and on day 55 proglottids were noted in the feces. On day 110 the gibbon appeared depressed and lethargic. By day 112 the gibbon had become comatose and euthanasia was performed. Necropsy revealed hundreds of 2-3 mm. cysts involving muscle tissue, brain, liver, lungs and spleen. One adult tapeworm approximately 60 cm. in length was removed from the small intestine.

The fungus previously isolated from gibbon skin lesions has been identified as an atypical strain of Microsporum canis. The organism is sensitive to griseofulvin. Infections continue to occur in the gibbon colonies principally at Phrabuddhabat, however response to treatment has been slow.

Summary: Animals produced (mice, rats, guinea pigs and hamsters) are available in sufficient numbers to meet planned or anticipated research requirements.

Mouse colony surveys show that GD VII, Sendai, and Reovirus 3 infections are common in the colony and that infestations with oxyurids and tapeworms occur. Corrective measures are being taken to improve the quality of mice issued to investigators.

A disease producing high mortality in suckling rats has been shown to be caused by ingestion of fine particulate sawdust in bedding material.

The gibbon (Hylobates lar) was shown to be a susceptable host of both adults and cysticerci of Taenia solium.

STUDY REPORTS

2. Title: "Ecology of the Tree Shrew (*Tupaia glis*)

Principal Investigator: Jack S. Stanton, MAJ, VC

Assistant Investigators: Prayot Tanticharoenyos, D.V.M.
Lenly D. Wetherald, SSG E6

Objective: The objective of this study is to evaluate the potential of the tree shrew for use as a laboratory primate and to colonize the species, define biological norms, determine prevalence of endoparasitic infestations and define bacterial flora of the intestinal tract.

Progress: During the period of this report 46 litters containing 86 tree shrews were born in the colony. Litter sizes varied between 1 and 3 with a mean of 2 and an average of 1.87 per litter. Of the 86 animals born 25 died or were killed by the parents prior to reaching weaning age.

18 tree shrews were issued to investigators in the laboratory during the year.

Because of increased demands for animal space and a lack of utilization of the tree shrew as an experimental model the efforts and space devoted to further work in colonizing the tree shrew have been limited.

Publication

Cadigan, Francis C., Jr., Stanton, Jack S., Tanticharoenyos, Prayoth, Chaicumpa, Verachat-The Lar Gibbon as Definitive and Intermediate Host of *Taenia Solium.* Journal of Parasitology, (in press).

SEATO MEDICAL RESEARCH STUDIES ON LEPTOSPIROSIS

Coordinator: Paul C. Smith, CPT VC, Asst Chief of Veterinary Medicine

Principal Investigators: Chua Wongsongsarn D.V.M.*
Paul C. Smith, CPT, VC
Jack S. Stanton, MAJ, VC
Achit Chotisen, D.V.M., Ph. D**
Richard O. Spertzel. MAJ, VC

Assistant Investigators: Dilok Kasornsombat, D.V.M.***
Preecha Klainil, D.V.M.***
Prayot Tanticharoenyos, D.V.M.
Jon H. Goodan, SP 4
Jack M. Preston, SP 4

Period of Report: 1 April 1966-31 March 1967

GENERAL INFORMATION:

Previous isolation and serologic evidence indicates a ubiquitous distribution of Leptospirae in Thailand. The prevalence of agglutinins in sera of domestic livestock continues to be very high even though clinical cases are seldom reported except in dogs. Surveillance of sera from domestic animal populations is continuing. The percentage of positive human sera tested in our laboratory continues to be low, contrary to reports from other investigators in this country. This laboratory has given active support to the virology department in studies on human sera taken from "Fever of Undetermined Origin" patients from The Republic of Viet Nam. Attempts to culture Leptospiral organisms from klong, paddy field, and river water and from animal tissues have resulted in some success.

* Chief, Education and Research Division (Dept of Livestock Development)
** Chief Immunology and Serology Branch (Dept of Livestock Development)
*** Department of Livestock Development.

STUDY REPORTS

1. Title: Serological Classification and Detection of Leptospirosis in Thailand.

Principal investigators:

Chua Wongsongsarn, D.V.M.*
Paul C. Smith, CPT, VC
Jack S. Stanton, MAJ, VC
Achit Chotisen, D.V.M., Ph.D**
Richard O. Spertzel, MAJ, VC

Assistant Investigators:

Dilok Kasornsombat, D.V.M.***
Preecha Klainil, D.V.M.***
Prayot Tanticharoenyos, D.V.M.
Jon H. Goodan, SP 4
Jack M. Preston, SP 4

Objective: The objective of this study is to determine the serological prevalence of leptospiral agglutinins in domestic animals and humans in Thailand.

Description: Single blood samples from domestic animals were collected by field teams of the Department of Livestock Development, Ministry of Agriculture. The sera were separated in the field by clot extraction and shipped to Bangkok with either wet or dry ice as a refrigerant. Other departments of the USA Medical Component, SEATO Medical Research Laboratory submitted paired human sera from FUO studies to be tested for leptospiral agglutinins. Fourfold serial dilutions were made and tested by the microscopic agglutination-lysis test utilizing eighteen different live diagnostic antigens.

Progress: During the report period, 1036 buffalo sera were collected and tested. Four hundred and twenty-nine, or 41.4% had titers of 1:25 or greater. One-hundred ten had their highest titers to L. hyos. The next most common serotypes appeared to be L. pyrogenes, L. borincana and L. wolffi. (See table). There were 808 cattle bled and tested, of which 312 or 38.61% gave positive reactions in the 1:25 dilutions or greater. Serological affinities for L. borincana, L. hyos, and L. wolffi were predominant. Sixty-two swine were tested and only 1 serum was positive. Sixteen pooled samples were taken from rodents in the laboratory animal production colonies and all were negative. In collaboration with the virology department, 585 human sera from the Republic of Viet Nam were tested with 41 showing reactions of 1:100 or higher. Three hundred seventy-eight other human sera were tested with only 2 positives. The predominant serological response in these human sera were to L. patoc, and L. borincana.

Summary: Serological evidence indicates that the prevalence of Leptospirosis in domestic animals is unusually high in Thailand. Though it is difficult to evaluate the clinical significance in animals it clearly indicates that domestic animals could prove to be a source of infection that might play a significant role in military medicine. The need for further study in the epidemiology of this disease is obvious.

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TABLE I

SERUM AGGLUTININ RESPONSES

Antigen	Buffalo (1036)		Cattle (808)		Human (RVN) (585)	
	1:25	1:100	1:25	1:100	1:100	1:400
L. patoc	19	0	26	1	9	10
L. butembo	13	3	4	0	0	0
L. celledoni	0	0	1	0	0	0
L. bataviae	6	0	2	1	3	0
L. pomona	3	0	2	0	0	0
L. djasiman	1	0	1	0	0	0
L. hyos	98	12	44	20	0	0
L. autumnalis	25	10	11	4	0	0
L. ballum	3	0	2	1		
L. canicola	38	11	16	4		
L. icterohmorrhagic	4	0	0	2	3	0
L. pyrogenes	53	12	7	3	2	0
L. alexi	0	1	0	2	0	0
L. grippotyhposa	3	2	2	1	1	2
L. borincana	44	14	63	58	5	1
L. wolffi	43	5	23	10	1	2
L. javanica	4	0	0	0	0	0
L. australis	1	1	1	0	2	0

2. Title: Isolation of Leptospires form Thailand, Modes of Transmission.

Principal Investigators:

Chua Wongsongsarn, D.V.M.*
Paul C. Smith, CPT, VC
Jack S. Stanton, MAJ, VC
Achit Chotisen, D.V.M., Ph.D**
Richard O. Spertzel, MAJ, VC

Assistant Investigators:

Dilok Kasornsombat, D.V.M.***
Preecha Klainil, D.V.M.***
Prayot Tanticharoenyos, D.V.M.
Jon H. Goodan, SP 4
Jack M. Preston, SP 4

Objective: The objective of this study is to evaluate by isolation techniques the role of wild or domestic animals and water in transmission of the leptospirae organism.

Description. Attempts are made to isolate leptospiral organisms either by intraperitoneal inoculations into weanling hamsters or by direct culture in rabbit serum enriched Fletcher's medium. Kidney plugs from aseptically exposed animal kidneys are taken with a pasteur pipette and discharged directly into tubes of Fletchers medium. Water samples collected from streams, klongs, and paddy fields are inoculated intraperitoneally into weanling hamsters. The animals are observed for 21 days for death or signs of illness. Those animals dying between 4 and 21 days post inoculation were cultured for Leptospirae. At least one animal from cages in which no animal was ill was cultured after 21 days.

Progress: Twelve isolates from rodent kidney punctures were serotyped as L. javanica. One isolate from a stream was identified as L. autumnalis. Thirty-nine water samples were taken from various provinces but only one from the six samples taken at Nakorn Nayoke was positive. Twenty-five urine samples from cattle and buffalo in that same area were all negative.

These samples were all taken during the dry, hot season of the year when the disease would be expected to be at its lowest prevalence. Experiments were conducted in an attempt to improve the isolation technique by concentration of dilute suspensions of viable organisms with millipore filters. All attempts failed to give positive results.

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SEATO MEDICAL RESEARCH STUDY ON MALARIA

Coordinators:

R.S. Desowitz, Ph.D., D.Sc.
F.C. Cadigan, Jr., MAJ, MC

Principal Investigators:

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Assistant Investigators:

Barnyen Permpanich
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SFC Harvey Gibson
Sahem Esah

Period of Report:

1 April 1966-31 March 1967

1 Preventive Medicine Officer, 9th Logistical Command (B)
2 Surgeon, 538th Engineer Bn.
3 Preventive Med Officer, 538th Engineer Bn.
4 Department of Pediatrics, Chulalongkorn Hospital, Bangkok
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General Information—Malaria

Malaria prevalence surveys. In response to specific requests in support of efforts of other departments malaria prevalence surveys were done in provinces of Thailand. In all case thick and thin blood films were examined after Giemsa staining. Due to differences in season of survey and in age distribution of population samples direct comparisons are not valid. In some cases pooling of data by province has obscured variations in point prevalence. These data are presented as being of general interest. Specific reference to these surveys may be encountered in reports of other departments.

Table I. Prevalence surveys for malaria in Thailand, by month, province and parasite species April 1966–March 1967

			Parasitemias					
		Number	P. falciparum		P. vivax		P. malariae	
Month	Province	Surveyed	No.	Rate (%)	No.	Rate (%)	No.	Rate (%)
Apr 66	Pathum Thani	433	1	0.2	12	2.7		
May 66	Lampoon	462	5	1.1				
	Chiengmai	67						
June 66	Yala	1167	108	9.3	21	1.8		
	Satul	612	12	2.0	4	0.7		
	Songkla	171	40	23.4	13	7.6	1	0.6
July	Pathum Thani	285	1	0.4	3	1.1		
	Korat	410	38	9.3	27	6.6		
Aug 66	Korat	72	39	54.2	19	26.4	—	—
Sept 66	Korat	103	42	40.7	25	24.3		
	Ubol	241	39	16.2	16	6.6		
Oct 66	Udorn	221	24	10.8	7	3.2	—	—
Nov 66	Cholburi	146	7	4.8	2	1.4	—	—
	Ayudhya	278	7	2.5	4	1.4	—	—
Dec 66	Kanchanaburi	374	16	4.3	4	1.1	1	0.3
Jan 67	Samut Songkram	166						
	PathumThani	2.5			3	1.4		
Feb 67	Pathum Thani	871	—		12	1.4	—	—
	Ayudhya	738	—	—	1	0.1	—	—
Mar 67	Nonthaburi	269						

Title: Field Trial of DDS plus Chloroquine–Primaquine as Malaria Prophylaxis

Principal Investigator: Phillip E. Winter, MAJ MC

Associate Investigators: Richard Auerbach, CPT MC*
Albert Nault, CPT MC**
M.R. Lewis, CPT MSC***

Period of Report 1 April–15 September 1966

Objective: To evaluate the efficacy of 4.4 diamino-diphenyl sulfone (DDS) in combination with standard chloroquine-primaquine in the prevention of malaria in U.S. Army personnel.

Description: Company C of the 538th Engineer Bn, camped and working in a malarious area south of Korat was studied. Personnel of the company were assigned to control or study group within the company, based on ASN terminal digit. The study group received a daily dose of 25 mgm DDS. The control group received a placebo identical to the DDS tablet, daily. Both groups were maintained on routine weekly doses of combined chloroquine-primaquine. Thick and thin blood films were obtained from each man prior to entry into the study and at monthly intervals thereafter. Dispensary personnel obtained similar films from individuals reporting on sick call with symptoms of fever, headache, backache and diarrhea. All films were stained with Giemsa and examined by personnel of SMRL. Records were kept of patients with clinical malaria or asymptomatic parasitemia, and results evaluated 6 months after initiation of the study.

Comment: This group of approximately 150 men was followed through the rainy season, when malaria cases were most likely to occur. A survey of indigenous civilians living in the area of the camp indicated that about half had *P. falciparum* infections, and that half of these were chloroquine resistant. During the period of study the malaria attack rate in the civilian population of the area was estimated to be 650/1000/annum. In the Royal Thai Army camp adjacent to the study group the attack rate was 300/1000/annum.

In this highly malarious setting, the U.S. Army engineer camp was a model of malaria discipline. The men lived in well-constructed screened barracks, and slept under bed nets. Underbrush was cleared in a wide radius around the camp. The camp was fogged with insecticide each night. An early curfew was imposed, to insure minimal exposure to the peak biting hours of *Anopheles balabacensis*, the presumed vector in the area. In addition to these measures, chloroquine-primaquine prophylaxis was employed.

Results: No cases of clinical malaria and no cases of asymptomatic parasitemia were noted in either control or study group. The study was accordingly terminated on 15 September 1966, although surveillance over the area has been maintained.

Summary: No cases of malaria were detected in a 6 month period in a U.S. Army Engineer Company, one-half of whom received daily DDS in addition to weekly chloroquine-primaquine, and one-half of whom received chloroquine-primaquine plus placebo. Malaria discipline in this group was rigorously enforced, and probably accounts for the absence of the disease in what must have been a highly malarious setting. Evidence as to the efficacy of DDS and/or chloroquine-primaquine as prophylaxis cannot be obtained from this data.

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Title: Study of Hemorrhagic Diathesis in Malaria

Principal Investigator: Pattrapon Bhanchet M.D., D.Sc.*

Associate Investigators: Francis C. Cadigan, Jr., MAJ, MC
Chulee Mitrakul, M.D., D.Sc.**
Supak Nueypatimanond, M.D.***

OBJECTIVE: Although purpura and other bleeding manifestations have been noted in malaria patients in the past, every few studies have been done on the pathophysiology of these disorders. A detailed study of clotting factors, disturbances in coagulability as well as other hemostatic mechanisms was instituted, using previously untreated patients with P. falciparum infections at Phrabuddhabat Hospital in central Thailand. Control subjects were relatives or friends of the malaria patients who had accompanied them to the hospital. Except that they were asymptomatic at the time of study, no health data are available on the control subjects.

PROGRESS: Forty-two malaria patients were studied on admission and twenty-six of these were studied at intervals throughout hospitalization. The patients were predominantly males (83%). Twenty-three control subjects were studied on whom 48% were males. The laboratory tests are incomplete at the moment but the following data are available.

Jaundice was noted in ten patients, palpable liver and spleen in twenty-four. The tourniquet test was positive in three patients (two male, one female) and bleeding occurred in five male patients (only one of whom had a positive tourniquet test). Of the five who bled, three had epistaxis, one had subconjunctival hemorrhage, one (with positive tourniquet test) had purpura and bleeding from the mouth. Approximately 25% were treated with chloroquine alone, 37% with quinine alone and 37% with chloroquine plus quinine.

The mean hematocrit of the patients is lower on admission than in the control group (Table 1). The mean value in patients continues to decrease through the fifth hospital day (Table 2). In only six of the forty-two patients did it rise by the fifth day of hospitalization.

An abnormal level of reticulocytosis was found in nine cases, most of which had marked anemia. Although the mean reticulocyte counts of the patients were within the normal range, 79% of the patients had a count greater than 0.1% compared to 26% of the controls. High reticulocyte counts were observed at the time of admission and they continued to rise throughout hospitalization.

Prolonged bleeding time was demonstrated in four out of forty-two cases; all four had marked thrombocytopenia. Venous clotting times were within the upper limit of normal.

A screening coagulogram has been done in ten cases of malaria and in eight controls (Table 3). There were abnormalities in the prothrombin time and partial thromboplastin time of patients but the thrombin time was within normal limits. Recalcification time were within normal limits. These data reflect defects in the first and second stage of the blood clotting mechanism, possibly in the prothrombin complex. Assay of the individual clotting factors is being done.

Indirect platelet counts were done on the first thirty patients and, after a phase microscope became available, direct counts were done on the other twelve patients. As can be seen from Tables 1 and 2, the

mean platelet count of the patients was markedly reduced on admission but increased rapidly into an almost normal range about one week after initiation of anti-malarial therapy. (Table 2).

Chemistry determinations have only been completed on eleven cases. Of these, seven had increase bilirubin (chiefly in the direct value), eight had increased thymol turbidity, only one had a slight rise in alkaline phosphatase, ten had a rise in SGOT, and three in SGPT. The levels of SGPT were considerably lower than those of SGOT.

Table 1

Hematology Values of Malaria Patients and Controls

	Male		Female	
	Control (11)	Patients (36)	Control (12)	Patients (8)
Mean values				
Hematocrit	38.4	33.4	35.3	29.1
Reticulocytes	0.1	0.49	0.15	0.56
Bleeding time	3'17"	4'23"	2'59"	5'19"
Clotting time	7'13"	8'16"	6'36"	9'6"
Platelets—indirect	510,000	212,000*	472,000	298,000***
—direct	261,000	77,000**	240,000	85,000****

* 27 patients
** 3 patients
*** 8 patients
**** 4 patients

Table 2

Mean hematology values during hospitalization of twenty-six malaria patients

	Hospital Day			
	1	2	4	8
Males (21)				
Hematocrit	34.5	31	28.2	26.4
Reticulocytes	0.49	0.54	0.57	1.1
Bleeding time	5'16''	4'51''	4'16''	3'54''
Clotting time	8'52''	8'34''	8'3''	7'37''
Platelets*				
indirect	167,000	167,000	197,000	443,000
direct	77,000	106,000	138,000	215,000
Females (5)				
Hematocrit	29.1	25.4	20.5	23
Reticulocytes	0.56	0.56	1.23	1.2
Bleeding time	6'15''	5'43''	4'25''	3'51''
Clotting time	10'8''	8'53''	8'37''	7'46''
Platelets**				
indirect	298,000	298,000	308,000	342,000
direct	85'000	89,000	115,000	237,000

* 13 studied by indirect method, 8 by direct method
** 2 studied by indirect method, 3 by direct method

Table 3

Mean Values of Screening Coagulogram

	Control (8)	Patient (10) Hospital Day			
		1	2	4	8
Prothrombin time	100%	64%*	71%	71%	79%
Partial thromboplastin time	81.5''	105.3''	95.4''	110.5''	98.4''
Recalcification time	91.0''	103.3''	107.7''	106.4''	88.0''
Thrombin time	3''	3''	3''	3''	3''

* 4 out of 10 cases had prothrombin activity below 60% of normal control.

Title: An Ovale-like Malaria Parasite of Man from Central Thailand

Principal Investigators: MAJ Francis C. Cadigan, Jr.
Robert Desowitz, Ph.D.

Associate Investigator: Sanit Puhomcharoen

During the course of a malaria survey of Lopburi Province, Central Thailand an unusual appearing plasmodium was found. The patient was a 22 year old Thai male farmer who complained of fever, chills and headache. After obtaining the blood smears he was treated with chloroquine to which there was a satisfactory response.

Description:

The parasite is unusual in that it exhibits the distortion of the host cell similar to that in P. ovale, and the schizonts, although containing a greater number of merezoites, are morphologicaly like P. malariae. However, while the host erythrocyte is stippled it is not as heavy as the Schuffner type, nor is there gross enlargement of the host cell, nor have band form trophozoites been observed.

Trophozoites

The youngest trophozoites seen are unexceptional. The round chromatin dot is prominent and there is a small solid block of well staining cytoplasm. The host cell is unaltered at this stage. Later ring stages occupy 1/3 to 1/2 of the erythrocyte. There is usually a single chromatin dot but double beads and accessory dots are not uncommon. Double infections are rare but present. The rings are regular and show no signs of amoeboidicity. No band forms were seen. Pigment is not discernible at this stage. The host cell is not stippled but may be enlarged and show elongated distortion.

In the slides available there were no forms intermediate between the ring stage and more advanced trophozoite. Because of this, the earliest from in which stippling of the host cell and presence of pigment occurred may not have been observed. The advanced trophozoite in which the vacuole is small or no longer present is irregular in outline but not markedly amoeboid. The cytoplasm stains a bright blue and there are fairly coarse deep gold pigment granules that have a blackish-brown cast. The nucleus is large and often has a distinct dark-staining nucleolus. The host cell stroma has practically disappeared and upon this almost colorless ground there is a distinct fine stippling that is lighter in color than that of the Schuffner type. The infected erythrocyte may or may not be enlarged but the majority show a P. ovale-like distortion.

Schizonts

The early schizont shows a tendency for the cytoplasm to round up. It occupies about 2/3 of the red cell. The pigment coalesces into coarse dark granules and at this stage has a tendency to aggregate along the periphery of the parasite. As schizogony advances a large amount of purplish staining material is seen to be interspersed in the blue cytoplasm. The mature schizont is round and rather P. malariae-like. It occupies 1/2 to 2/3 of the erythrocyte which is usually enlarged. There is a central mass of dark blackish-brown pigment. There are 12 to 16 merezoites.

Gametocytes

The gametocytes are round and contain numerous rather coarse pigment granules. The mature gametocyte occupies almost the entire host erythrocyte.

Discussion

Diagnosis of this parasite is difficult. It has been shown to Dr. John W. Field who thought it an aberrant P. vivax and to Dr. R.S. Bray who felt it was an aberrant P. malariae. Field in his book on microscopic diagnosis of malaria (Part 2, 1956) notes that ovale-like vivax has been reported particularly in individuals in chronic or chemotherapeutically partially suppressed infections. As far as can be ascertained our patient had not taken anti-malarials prior to the making of the blood films. Unlike classical P. ovale, the stippling of the host cell is finer and lighter. Also the first appearance of stippling and pigment occurs at a later stage of development than P. ovale. The only simian malaria it might resemble is P. fieldi but does not show the intense heavy eosinophilic stippling of that parasite.

Further surveys are to be carried out in the area to attempt detection of other cases with this parasite. At this time no diagnosis as to species can be given.

Title: Hepato-renal Changes in Blackwater Fever

Principal Investigator:	Narong Sadudee, M.D.
Associate Investigators:	Visitr Benjapongse, M.D. Natth Bhamarapravati, M.D. Kasem Angustasin, M.D. Francis Cadigan, Jr., Maj. MC
Period of Report	15 May 1966 - 31 March 1967

Only 3 kidney biopsies since the project was implemented were obtained. This is due to the fact that Dr. Visitr could not obtain biopsy needles which have been requested repeatedly since the beginning of the project. Conclusion is not available at this time.

Title: Host-Parasite Relationships in Malarias

Principal Investigators:	Robert S. Desowitz, Ph.D. D.Sc. Francis C. Cadigan, Jr., MC, MAJ Louis H. Miller, CPT MC Katchrinnee Pavanand, M.D. Bernhardt W. Langer, Jr., Ph.D.
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Assistant Investigators:	Barnyen Permpanich MSG Yael Friedlander Duangduen Vacharaphorn SFC Harvey Gibson

Objective — A broad study has been undertaken to investigate the many factors contributing to host-parasite relationships in malarias. During this year investigations have been carried out on the pathophysiology and immune response of the host and the physiology of the parasite itself. Since there are many species of malaria parasites, each with its own host-parasite relationships the overall approach to this long-term program has been a comparative one. This year's report encompasses a descriptive account of host pathophysiology and some aspects of immunity (immunoglobulin and other serum protein alterations) in mesoendemic malaria in humans, in P. falciparum in gibbons, in a natural malaria of gibbons, in P. coatneyi and P. inui in rhesus monkeys. Renal pathophysiology of P. berghei in mice has also been studied. The investigations now in progress are designed to elucidate the mechanisms of some of the physiologic derangements observed in the various hosts. For example, blood volume is being studied in P. coatneyi infections during the haemoconcentration and haemolytic phases. Cholesterol metabolism in infected gibbons is being studied to explain the marked hypocholesterolaemia of that infection. A series of papers under the general title of "Comparative Studies in the Pathology and Host Physiology of Malarias" has been or is being submitted for publication.

The investigations on parasite physiology is also of a comparative nature. This year's work on the pentose cycle in P. berghei has been completed. Much of the time has been occupied in working out a suitable methodology for these studies. Work now in progress is concerned with metabolic processes of primate and human plasmodia.

Because of the rather broad diversity of this program the report is presented as a series of separate but interelating investigations under their subtitles.

Subtitle: A Preliminary Study of Diagnostic and Clinical Problems Associated with Mesoendemic Malaria in Southeast Thailand.

Investigators: R.S. Desowitz and L.H. Miller

A long-term longitudinal community-level study of malaria is planned. Preliminary to this, an investigation was carried in a mesoendemic area of Trad province, S.E. Thailand in order to determine some of the problems that might be encountered and to clarify the objectives of the larger study. In addition to the logistical and community relationship problems of such a study, information was sought on such factors as (1) the best method for microscopic detection of the parasite, (2) the correlation between presumptive clinical diagnosis and microscopic confirmation and (3) serum biochemical changes in the mild to moderate infections that would not normally warrant hospitilization.

Methods

Because of the brief time available a comprehensive epidemiologic study was not undertaken. At the community level, fever cases and controls were examined and a small survey of 61 schoolchildren carried out in two schools. The children in the first school were selected for those who the teacher said had not been feeling well and in the second school those that had an enlarged spleen as well as a history of chronic illness. In the village study a clinical work-up was obtained on 36 fever cases. The blood films were examined immediately and from those found positive, blood was drawn for serum chemistries (see Table II). Serum from controls was obtained at the same time. The clotted blood was centrifuged in the field and sera samples frozen in dry ice. The chemistries were carried out within a week of collection. For the schoolchildren only blood films and examination for splenomegaly were carried out.

Results and Discussion

1. Spleen rate in schoolchildren

Five of 26 children from the first school had an enlarged spleen. If this spleen rate in children was confirmed in a larger and unselected survey, then this area can be considered mesoendemic for malaria.

2. Parasitologic diagnosis.

For many years the accepted method of detecting plasmodia in low-density parasitaemias has been by examination of the thick blood film. Recently Dowling and Shute (1966) have questioned the efficacy of this technique. They reported that 60% to 90% of the parasites may be lost during dehaemoglobinization. Thus a thorough search of the thin film may reveal more scanty infections than thick film examination. These factors may be of extreme importance in detecting cases during the consolidation phase of malaria eradication as well as in confirming cases only clinically suspected to be malaria. For example, our examination of the records at Trad Provincial Hospital showed that on the average, 400 cases of malaria are treated each month although only about 100 of these are confirmed by blood smear.

In this present investigation a total of 82 smears (thick and thin) were obtained and stained by Romanowski's rapid method. Each slide was examined by three experienced microscopists. The first examination was made in the field with a search of the thin film only for 5 minutes. The second examination was in the laboratory with a thin film search of 20-30 minutes and independent confirmation of each positive. The third examination was carried out by a former Supervisor Microscopist of the Thai NMEP by searching the thick smear for 5-10 minutes (for method of detection used by the NMEP). The results are summarized in Table 1. It will be seen that the field examination of the thin films was as good or better than that of the thicks. The 20-30 minute search of the thin films was strikingly superior and revealed at least twice the number of parasitemias. Of the 20 positives 2 were P. vivax and these were picked up by all three methods.

However, gametocytes were found more frequently by thick smear examination (6 positive for gametocytes by thick smear and 4 positive by 20 minute thin smear examination).

Future study should resolve the question of what is the best method of examinotion for different purposes, e.g. survey, case finding, and detection of drug resistance. The problem of gametocyte carriers also deserves study. In this survey all were children (P. falciparum gametocyte carriers). Does this age group constitute the main reservoir in this region? What gametocyte density and other factors are involved in infecting the mosquito host?

3. Clinical Diagnosis of Malaria: Of the 36 patients evaluated clinically, 18 were felt to have malaria on the basis of fever, chills, headache anorexia, nausea, and/or orthostatic hypotension. Eight out of these 18 had positive smears for malaria. A negative malaria smear in those patients with findings suggestive of malaria may be explained by treatment with antimalarial drugs, parasites too scanty on the day of study or other diseases mimicking malaria. The finding that of 18 patients considered to have a disease other than malaria 4(3 with URI, 1 with mumps) had circulating parasites is disturbing and raises the possibility that presumptive treatment should be given to a larger group of patients, accepting that many will eventually prove not to have malaria. Formulation of the problem: In an area of known chloroquine-resistant falciparum malaria, where presumptive therapy is probably inadequate and may potentially increase the problem of chloroquine resistance how can the case identification best be carried out? Radical treatment may have to include drugs such as quinine and because of expense and side effects would necessitate good case identification. Possible questions to be answered in the field:

1) How many patients with the clinical diagnosis of malaria but with negative smears will prove to have malaria if smears are taken on subsequent days?

2) How many patients with clinically identified diseases other than malaria, actually have malaria either alone or in addition to the other disease?

3) In a community subject to mesoendemic malaria and where the majority of infected individuals have scanty parasitemias what is the rate of asymptomatic infections? At what rate and under what conditions do the asymptomatic convert to a clinically active disease?

4) What is the response of falciparum malaria to presumptive and radical treatment with chloroquine in this area?

4. Serum Biochemical Changes: Other studies of serum biochemical changes in malaria have usually studied a hospitalized population. Our aim was to evaluate changes resulting from a milder disease. The only significant changes caused by mild malaria were a lower serum cholesterol and albumin (Table II.) These changes have been observed in more severe malaria and in animal malarias.

The etiology of these abnormalities has not been studied. The serum sodium, potassium, and chloride were all normal, whereas in more severe disease they are low. One patient with vivax malaria had an elevated BUN (27.5 mg/100 ml), creatinine (1.5 mg/100 ml) and SGPT (51. S.F. units). One patient with falciparum malaria had an elevated SGOT (64 S.F. units). However because these values were normal in the great majority, the averages were not significantly different. In this small sample it would not be valid to necessarily attribute these individual changes to malaria. The gamma globulin was not significantly different, although in other studies it has been shown to be elevated. It may be that in an endemic area the level of gamma globulin is high in the entire population as shown in this small sample.

Table I – The readings on 82 malaria smears by 3 methods.

	Smear +	Smear –	% positive
Field examination of thin smear for 5 minutes	12*	70	15
Thick smear for 5–10 minutes	7*	75	9
Thin smear for 20 minutes	20*	62	24

* Two patients by each method had P. vivax. The rest were P. falciparum.

TABLE II – The Comparison of Average Serum Biochemistries between Malaria Patients and Healthy Controls in a Thai Village.

	Malaria Patients	Healthy Controls	"p" Value
Number	10	7	
Average age	30.7	33.6	
Hematocrit (%)	40.3	41.4	N.S.
Serum Sodium (mEq/L)	138.7	140.6	N.S.
Serum Potassium (mEq/L)	3.94	3.89	N.S.
Serum Chloride (mEq/L)	102	103	N.S.
Serum Creatinine (mg/100 ml)	0.92	1.0	N.S.
BUN (mg/100 ml)	13.1	10.8	N.S.
Serum Cholesterol (mg/100 ml)	126	159	<0.02
SGOT (S.F. Units)	30.7	27.0	N.S.
SGPT (S.F. Units)	18.3	14.9	N.S.
Serum Alkaline Phosphatase (Sigma Units)	3.33	2.30	N.S.
Thymol Turbidity (Units)	7.23	8.43	N.S.
Bilirubin – Total (mg/100 ml)	0.55	0.40	N.S.
Direct (mg/100 ml)	0.11	0.16	N.S.
Total Serum Protein (g/100 ml)	7.45	7.46	N.S.
Serum Albumin (g/100 ml)	3.36	3.68	<0.01
Serum α_1 Globulin (g/100 ml)	0.32	0.26	N.S.
Serum α_2 Globulin (g/100 ml)	0.59	0.59	N.S.
Serum β Globulin (g/100 ml)	0.85	0.84	N.S.
Serum γ Globulin (g/100 ml)	2.39	2.09	N.S.

Subtitle: Plasmodium falciparum in the Gibbon (Hylobates lar lar). Pattern of infection.

Investigators: MAJ Francis C. Cadigan, Jr., MC
Verachat Chaicumpa, DVM
Sanit Puhomchareon

The white-handed gibbon (Hylobates lar lar) of Thailand has previously been reported to be susceptible to both blood and sporozoite induced infection with P. falciparum. This paper reports more prolonged observation of infections, and the influcence of factors such as multiple passage, blood types and intercurrent infections on the pattern of parasitemia in splenectomized gibbons.

METHODS

The animals used were juvenile white-handed gibbons (Hylobates lar lar) from Thailand. All animals were splenectomized and treated with chloroquine and primaquine. No animals had detectable parasitemia before or after surgery.

Blood grouping was done with human anti-A and anti-B sera. Previous studies by Wiener et al. (1) habe shown that the gibbon erythrocyte A and B antigens, while not identical with the human antigens are quite similar and that they react with human antisera. Crossmatch was done in the major mode (human cells into gibbon sera) and without the addition of albumen.

Blood smears were made daily and stained with Giemsa stain. Parasite counts were made in terms of number of trophozoites or gametocytes per 500 white blood cells and converted to number per cubic millimeter by using the mean white blood count.

RESULTS

Effect of blood group compatibility. Of eighty-four gibbons tested for the ABO blood group, none were group O, twenty-two were group A, thirty-six were group B and twenty-six were group AB. Crossmatches between human erythrocytes and gibbon sera of the same blood group were grossly incompatible. Similarly crossmatches between human O negative cells and gibbon sera of the three different groups (A,B,AB) were incompatible on gross examination. Thus there was no evidence to support the possibility that there was a prolonged survival time of human erythrocytes in the gibbon and that prolonged survival was the reason for success in transferring P. falciparum infection from humans to white-handed gibbons. Despite the incompatibility of human cells with gibbon sera, the fourteen attempts to pass P. falciparum by blood transfer from humans to gibbons were all successful.

On the other hand, in the gibbon to gibbon transfers there is evidence that blood group compatibility has an effect on the speed with which peripheral parasitemia becomes patent and upon the speed with which the initial peak is reached. Table 1 shows that although all transfers were successful and all reached a parasitemia level of 1% in the first month when there was transfer from an infected gibbon into recipient gibbon which had an incompatible group (e.g. A into B or AB into A) there was a delay in the mean prepatency period and a delay in reaching the 1% parasitemia level. It can be seen that this was not so great as to be of significance in the selection of animals.

Effect of inoculum size. It is also apparent from Table 1 that the size of the inoculum is of at least equal importance as the compatibility of blood. In one instance in which an identical inoculum was put into five gibbons simultaneously, patency occurred in the compatible recipients on days 4, 6 and 10, and in the incompatible recipients on days 4 and 6. Peaks occurred in the compatible animals on days 21,20, and 31 respectively, cnd in the incompatible animals on days 26 and 21 respectively.

Duration of infection. Peripheral parasitemia is usually detectable throughout the first eight to ten weeks of infection and thereafter is variable in occurrence. Parasitemia may not be detectable on peripheral smear for several weeks or even, less often, several months and then reappear. The mean overall duration of detectable parasitemia is thirty-one weeks or about seven months. In individual animals it has varied from as short as six to as long as seventy-two weeks. In the two strains which have undergone multiple gibbon passage, there has as yet been no indication that the duration of infection was affected. One of these, strain B, is now in its twenty-second gibbon passage but only the first eleven passages have been followed for a sufficient period to comment on duration of the infection. Strain A in four passages has not changed its mean duration.

Pattern of infection. Five gibbons which were inoculated simultaneously with the same dose of P. falciparum (strain A) had similar parasitemia curves. These are shown in figure 1. It can readily be seen that the basic pattern is the same in each animal. As infection continued, succeeding periods of high peripheral parasitemia tended to be of shorter duration, to occur at greater intervals and to attain lower maximum levels.

Intercurrent infection. Naturally occurring upper respiratory infection, herpes simplex infection and superficial fungal infections have not affected the levels of parasitemia, nor have they resulted in malaria disease. Gibbons inoculated with dengue types 1 and 2 virus, although showing evidence of infection (serologic changes or viremia), did not show alteration of the pattern of the malaria infection.

Effect of multiple passage. There is no evidence in the twenty-second passage of one strain nor in the fourth or fifth passage of two other strains that there is any great alteration in the pathogenicity of the parasite. No disease symptoms attributable to malaria infection have occurred even in the presence of a 8% parasitemia. No significant change in the time before patency nor before reaching a 1% parasitemia level has been noted. Chloroquine resistance has been maintained through twenty-two passages. As mentioned previously, the duration of infection has not been altered by multiple passage.

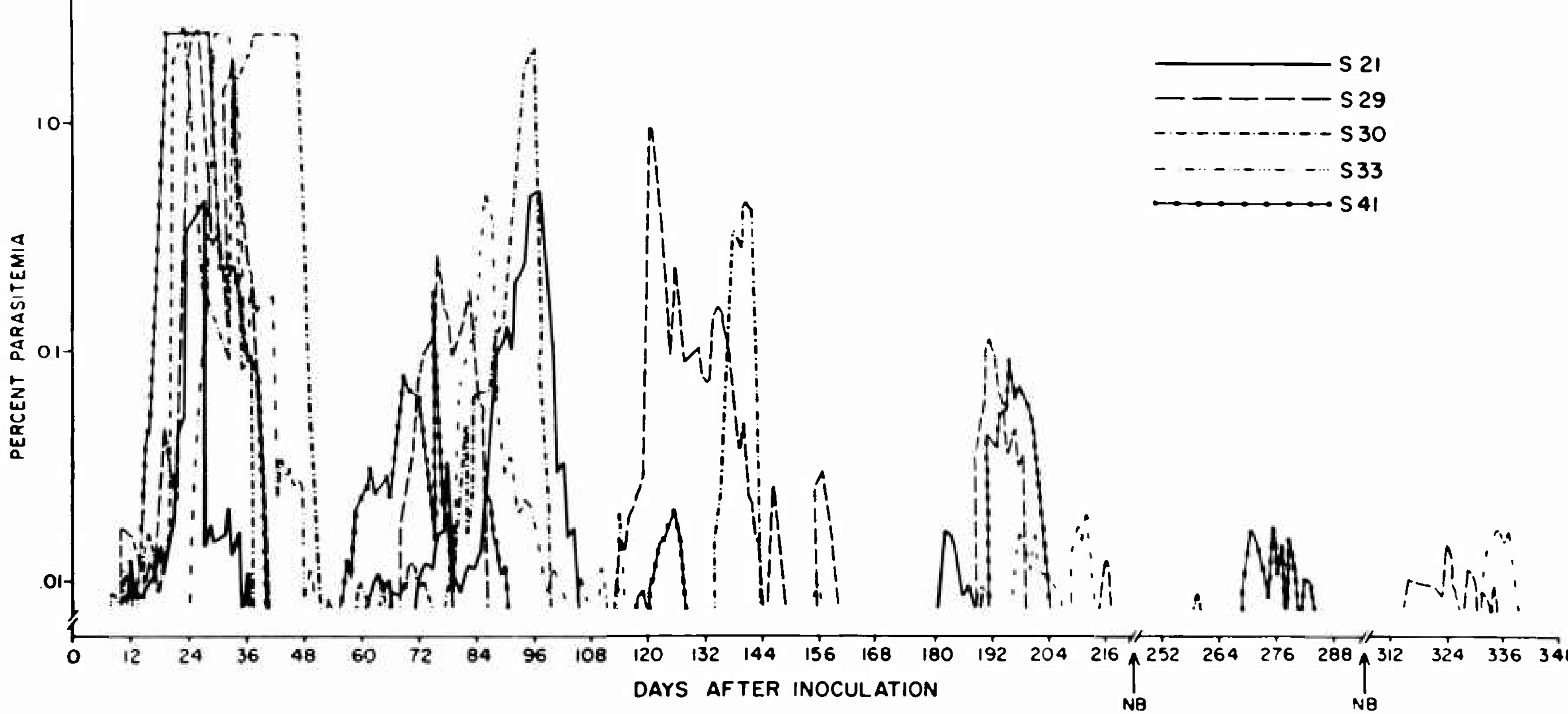
The effect of multiple passage on gametocytemia seems to be an increase in the number of gametocytes and the number of days on which they are found. Precise quantitation of this has not been done yet, but a definite trend is obvious. In addition, whereas the gametocytes in early passages were all very immature, now many of them are more nearly mature and on two days in the 14th passage, a few morphologically mature gametocytes were seen. Attempts to infect mosquitoes from gibbons have been unsuccessful. Details are listed under another study report. The frequency with which schizonts are noted in the peripheral blood has increased markedly.

Table 1. Effect of ABO compatibility and size of inoculum on infection

Compatible			Incompatible		
Patent on Day	1% Parasitemia on Day	Inoculum* Parasites x 10^6	Patent on Day	1% Parasitemia on Day	Inoculum* Parasites x 10^6
1	3	300	1	6	300
1	3	300	1	6	300
1	4	300	1	10	300
1	4	300	2	6	300
1	4	150	5	15	15
1	5	300	6	13	40
1	18	300	6	15	150
2	5	300	6	25	50
2	9	40	7	14	300
2	18	300			
3	8	40			
3	10	60			
6	31	1			
Mean 1.9	9.4		3.9	12.2	

* Numbers have been rounded off for ease in reading table.

FIG I COMPARISON OF 5 GIBBONS INOCULATED SIMULTANEOUSLY



Subtitle: Effect of P. falciparum Infection on Serum Biochemistry Values of the Gibbon

Investigators: MAJ Francis C. Cadigan, Jr., MC
Verachat Chaicumpa, DVM
Sanit Puhomchareon

CHEMISTRY

Since white-handed gibbons (Hylobates lar lar), unlike chimpanzees continue to have intermittent parasitemia for as long as twelve moths after P. falciparum infection produced by blood inoculation and most can be expected to have recurrent high peaks of parasitemia for at least three to four months and since there is no disease produced and thus no treatment required to keep the animals alive, it was felt that this animal would make a good model for study of the long term effects of P. falciparum on the serum chemistries.

METHODS

Ten splenectomized juvenile white-handed gibbons (Hylobates lar lar) were inoculated with P. falciparum which had been maintained by blood passage in gibbons for approximately fifteen months. Gibbons P10,P11,S1,S13,S23,S25,S30, and S67 all received sixteenth passage material. Gibbon S77 received seventeenth passage material (from S67) and gibbon 76 received twentieth passage material. All inoculations were with fresh heparinized blood and the dose was approximately 10^{n} parasites. The donor animal for sixteenth passage material was blood group A, the recipients were AB,B,AB,B,B,B,B,B, respectively. The donor for S77 was group B; gibbon S77 is group A. The donor for S76 was group A; gibbon S76 is also group A.

Gibbon P10,P11,S1 and S30 had been previously inoculated successfully with other strains of P. falciparum. Gibbons S23 and S25 had been inoculated with blood from two patients who were originally thought to have P. vivax infections but were subsequently appeared to be mixed infection with P. vivax predominating. These gibbons are described in detail elsewhere.

After inoculation the animals were examined daily for evidence of overt disease and, for the first sixty days of infection, rectal temperatures were taken daily. Thick and thin smears of peripheral blood were made daily and stained with Giemsa stain. Parasites counts were recorded in terms of the number of trophozoites per 500 WBC. Blood was drawn at 7 day intervals for determination of hematocrite, BUN, cholesterol, bilirubin, thymol turbidity, alkaline phosphatase, SGOT, SGPT, creatinine, total protein and electrophoresis. Blood chemistries were done in the same manner as described by Desowitz et al. in another report in this series. Studies on S76 and S77 were for a period of three months, the remainder were studied for six months.

RESULTS

All infections were patent on peripheral blood smear by day 6 (Table 1) and reached a 1% parasitemia level by day 21. All of those followed for six months except P10 continued to show significant parasitemia levels for at least 120-days. Figures 1 and 2 show representative patterns of the biochemical changes compared to peripheral parasitemia.

At no time in the course of infection did any gibbon show evidence of any symptoms attributable to malaria. No change in behaviour, appetite or attitude was noted. Minor fluctuations in body temperature occurred as was reported in a previous series but no clinically significant correlation could be made with the stage or degree of infection.

TABLE 1

	First patent	Parasitemia over 1%	No of parasites in inoculum
P10	Day 2	Day 8	12.8×10^7
P11	Day 2	Day 8	12.8×10^7
S1	Day 1	Day 7	12.8×10^7
S13	Day 1	Day 6	12.8×10^7
S23	Day 1	Day 18	12.8×10^7
S25	Day 1	Day 8	12.8×10^7
S30	Day 2	Day 21	12.8×10^7
S67	Day 2	Day 4	9.6×10^7
S76	Day 6	Day 13	6×10^7
S77	Day 6	Day 15	18×10^7

In every instance, a drop in hematocrit followed peaking of peripheral parasitemia. The lowest hematocrit noted was 28% in S1. Detailed analysis of hematologic response to falciparum infection in the gibbon is given elsewhere.

The blood urea nitrogen, direct and total bilirubin, thymol turbidity, and creatinine levels fluctuated during the observation period but showed no correlation with parasitemic curves and no positive trend except that the BUN increased in the first two weeks after infection in all animals and showed an overall tendency to continue to rise in gibbons P11, S13, S25, S30 and S67.

Cholesterol levels dropped consistently, at about the same time that the hematocrit fell, to levels as low as one-third of pre-infection levels. Although the serum cholesterol dropped with subsequent rises in peripheral parasitemia, the decrease was not as marked as on the original parasite peak.

The transaminase levels showed no significant fluctuation in the six gibbons which had been infected previously, but marked increases in both SGOT and SGPT occurred in the four animals which had not been inoculated with malaria previously. There was a lag of at least several days after peak parasite levels were attained before the enzyme values increased. Since determinations were done at 7 day intervals, it is not possible to define the response further at this time.

Seven of the ten animals showed a slight but definite rise in total protein during the study period. Serum albumen levels showed a consistent rise from pre-infection levels in the first few weeks and then remained fairly constant. Globulin levels decreased slightly in most of the animals during the first month and then gradually increased. The A/G ratio increased during the first few weeks of infection, then gradually fell to slightly higher than pre-infection levels.

In the globulin fractions, there was no major change or trend in the alpha-2 globulin. Beta globulin decreased slightly during the first ten days in six animals and then with minor fluctuations remained at the same level. Gamma globulin showed a slight decline in the first ten days in all but two animals, then in six animals began to rise after 40 days and remained stabilized after 100 days. Two animals had transitory rises at 70-80 days. In most instances the gamma globulin increased 50-100% above pre-infection levels.

Alkaline phosphatase levels decreased in six cases, rose in three cases and remained essentially unchanged in two. Two of the six cases which showed a rise (S76 and S77) had very marked rises with a peak in the third week followed by a drop to normal by the fifth week.

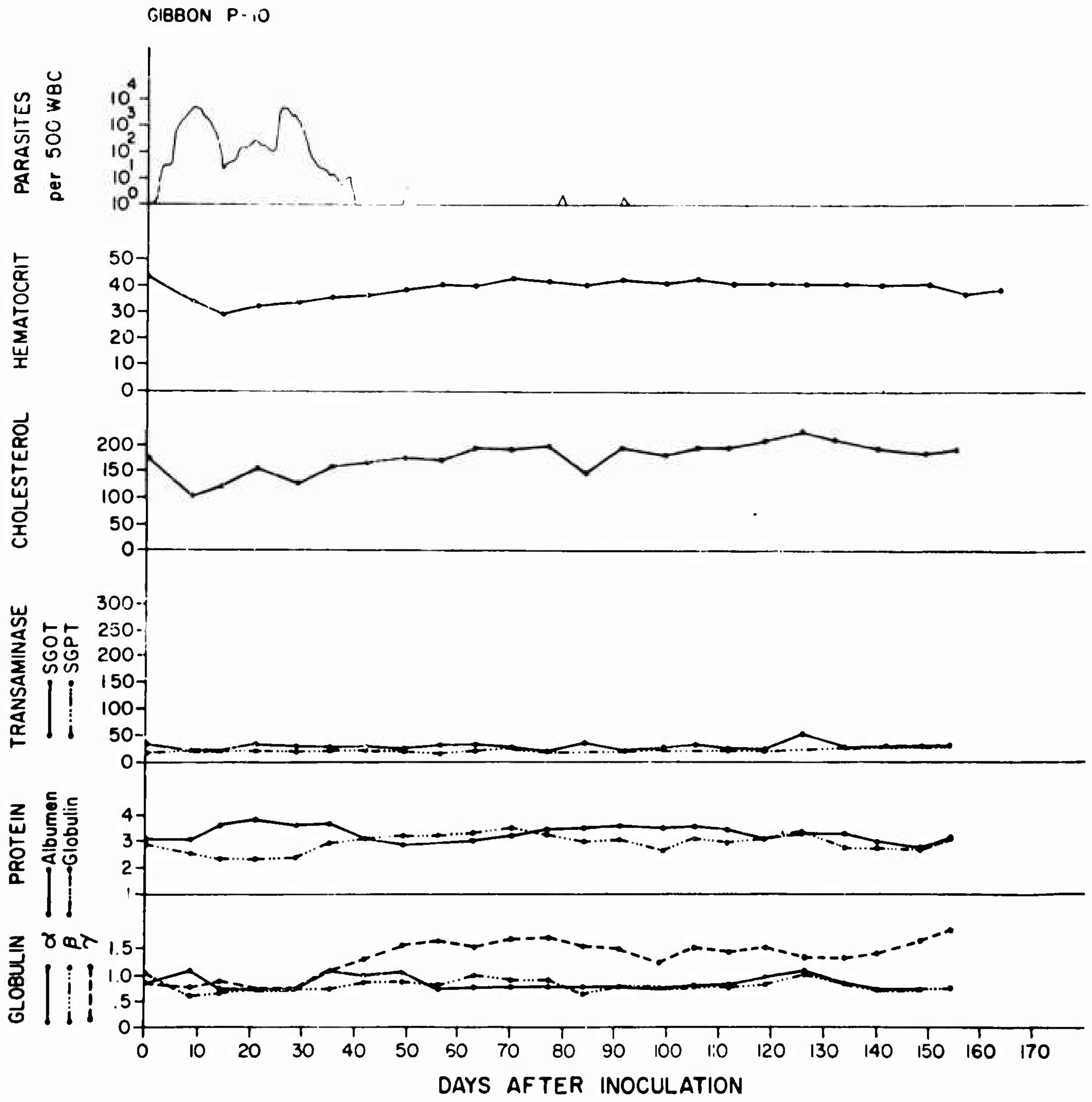


FIGURE I. COMPARISON OF HEMATOCRIT AND SERUM CHEMISTRY VALUES WITH PERIPHERAL PARASITEMIA IN A GIBBON PREVIOUSLY INFECTED WITH AN HETEROLOGOUS STRAIN OF *P. FALCIPARUM*.

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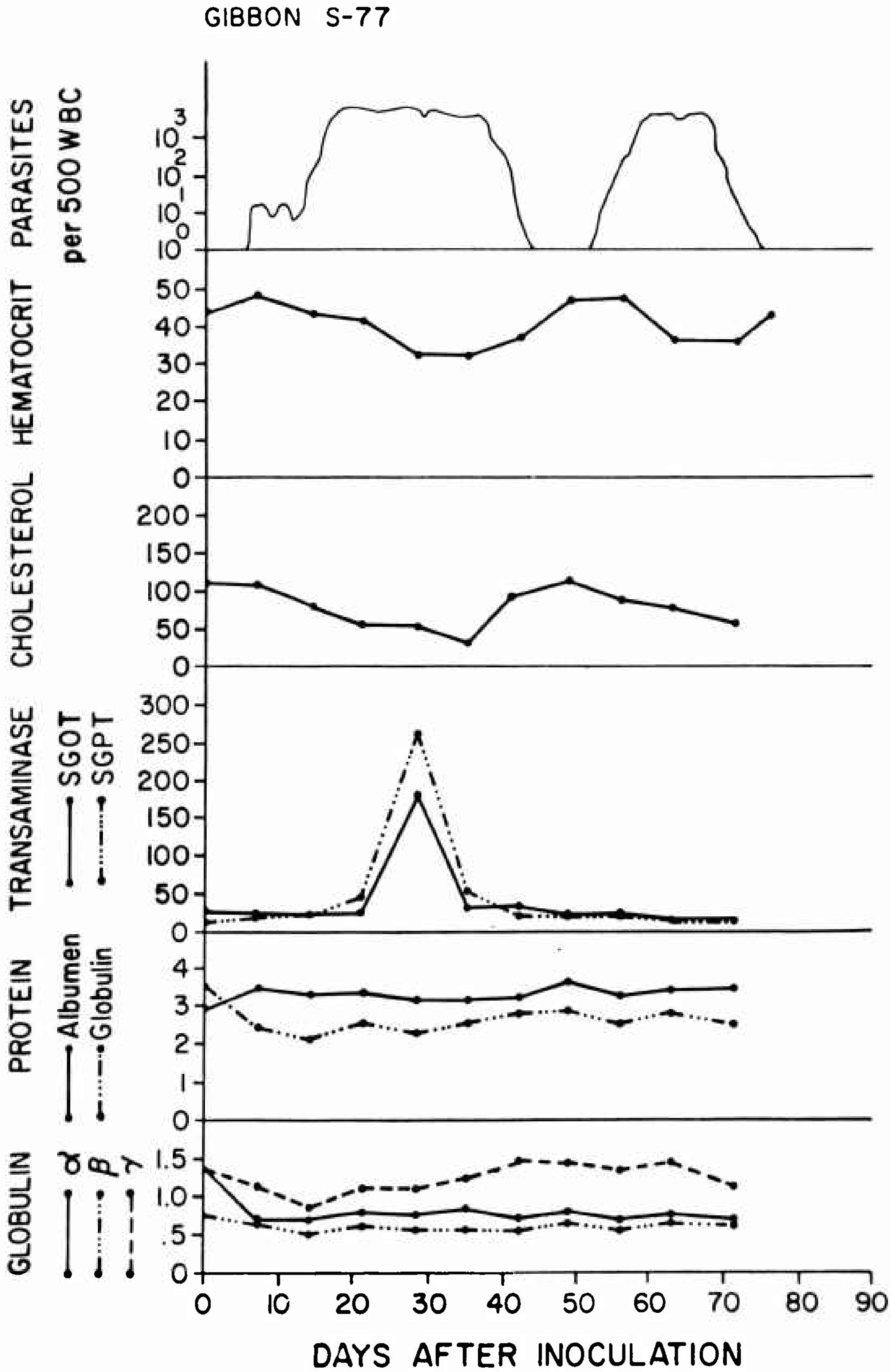


FIGURE 2. COMPARISON OF HEMATOCRIT AND SERUM CHEMISTRY VALUES WITH PERIPHERAL PARASITEMIA IN A GIBBON IN ITS FIRST INFECTION WITH P. FALCIPARUM.

Subtitle: Protection Afforded by Previous Infection with Homologous and Heterologous Strains of *P. falciparum*

Investigators: MAJ Francis C. Cadigan, Jr. MC
Verachat Chaicumpa, DVM
Sanit Puhomchareon

Previous studies in chimpanzees have shown that a South East Asian strain of *P. falciparum* would not protect against infection with a West African strain of the same parasite. Since the effectiveness of any attempts at active immunization would be dependent upon the homogeneity of the strains in the area where immunization would be done, we attempted to determine the degree to which several isolates of *P. falciparum* from a relatively small area would show cross-protection.

METHODS

The isolates of *P. falciparum* were obtained from patients at the Phrabuddhabat Hospital and the nearby Passive Detection Center of the National Malaria Eradication Project (Region I) located in Saraburi province in Central Thailand. The exact locations of the patients homes are not known, but are all from the general area from which patients are drawn or roughly from 40 miles north to 20 miles south along the mountain range in which these institutions are located. All of the original infections in gibbons were started as blood transfers from the human cases except P10, S1, S3 which were sporozoite induced infections. Subsequent gibbon passage was by blood transfers. Each isolate in gibbons was given a letter designation. Table 1 lists the previous infection histories of the gibbons which had been previously infected. Animals P1, P2, P3, P7, P10, P13, S2, S10, S12, S30 had reached a 1% level of parasitemia at some time during their infection.

Animals used were juvenile white-handed gibbons (*Hylobates lar lar*) which were wild-caught in Thailand. After a period of adjustment to captivity, all animals were splenectomized and then treated with 17.5 mg/kg chloroquine and 10.5 mg/kg of primaquine. No malarial parasites were observed before or after surgery in any of these animals. Before challenge with a second infection of *P. falciparum,* all animals had been free of peripheral parasitemia for at least several months.

Blood smears, both thick and thin, were done daily on all gibbons and stained with Giemsa stain. Parasite counts were made on the basis of the number of trophozoites per 500 white blood cells and converted to number of parasites per cubic millimeter by using the mean white blood count.

The following schemata of challenge were used.

1. In test I, isolate B was used to challenge an animal previously infected with isolate B (P3) and a virgin animal (S39).

2. In test II, isolate B was used to challenge animals previously infected with isolate B (S2), A (S7) J (S10).

3. In test III, isolate B was used to challenge one animal with two prior inoculations of A (P1), one animal with two prior inoculations of B (P3), one animal with a single inoculation of B (P2) and two virgin animals (S59, S60).

4. In test IV, isolate B was inoculated into animals which had previously been infected with A (S30), B (P7), C (P11), D (P13), F (P10), G (S1), H (S3), L (S12), N (S25), O (S23), and S13 (a virgin).

5. In test V, isolate A was used to challenge an animal infected with A (S41), one infected with B (S60), one which was infected twice with B (P2), one which had had three inoculations of B (P3), an animal which had had Q (S58) and a virgin animal (S79).

TABLE 1

	Isolate	Gibbon Passage	Number of weeks of parasitemia	Remarks
P1	A	First/Third	27/9	Rechallenged
P2	B	Second	28	
P3	B	First	36	
P7	[illegible]	First	54	
P10	F	First	12*	Sporozoite induced
P11	C	First	37	
P13	D	First	37	
S1	G	First	14*	Sporozoite induced
S2	B	Second	27	
S3	H	First	14*	Sporozoite induced
S7	A	Second	27	
S10	J	Second	14	
S12	L	First	23	
S13	—	—	—	
S23	O	First	12	
S25	N	First	19	
S30	A	Third	21	
S39	—	—	—	
S41	A	Third	41	
S58	Q	Second	19	
S59	—	— —	—	
S60	—	—	—	

* End of observation period, full duration not known

RESULTS

The parasitemia curves are shown graphically in figures 1-5. It can easily be seen in each test that prior infection with the homologous isolate confers a marked degree of protection upon challenge. Although re-infection occurs, the parasite levels are lower by 1-2 logs than the control and in the two tests in which there was a long follow-up, the total days of detectable parasitemia differed markedly as well.

In the three tests in which animals previously infected with isolate A were challenged with isolate B there was evidence of partial protection as shown by a delay in reaching high levels (1% parasitemia) compared to the controls. This was particularly marked in the case of P1 (Fig 3) which had been infected twice with isolate A before challenge with B. Conversely, an animal previously infected with isolate B (S60 in Fig 5) showed a delay in building up the level of parasitemia when challenge with isolate A. It is interesting to note that P2 which had had two infections with B showed a greater degree of protection to isolate A than did S60 and that P3 which had had three previous infections with B showed as much protection as did S41 which had been infected with the homologous strain.

From Figure 4 it can be seen that infection with isolates C, G, N. and O conferred no protection against challenge with isolate B. Gibbon S3 which had had previously been infected with isolate H showed the same response to challenge with B as did P7 for which B was an homologous challenge. It is possible that these two isolates are an identical strain. Isolate F(P10) did not confer any protection in the first 30 days but may have done so subsequently. An intermediate type of response was shown by gibbons P13 and S12 (isolates D and L which showed protection of over 1 and 0.6 logs respectively but which continued to have low levels of parasitemia for a prolonged period.

From these data we would surmise that at least four strains are represented here by isolate A, B and H, D and L, and the group C, G, O and N. The latter group could easily represent more than one strain, of course, since they are grouped on the basis of no protection against B. The results with isolate F are such that no relative position in terms of the other isolates can be assigned. Isolate J (Figure 2) while dissimilar to A and B was not tested in comparison with the other strains and although its pattern resembles that of P7, no prediction of relationship can be made from these data.

In summary, one can say that of eleven isolates compared with B, only one (H) gave cross-protection of a degree similar to the homologous isolate and only two (D and L) gave persistent partial protection. Isolates A and F delayed the peak of parasitemia which possibly could be of value as an adjunct of chemotherapy.

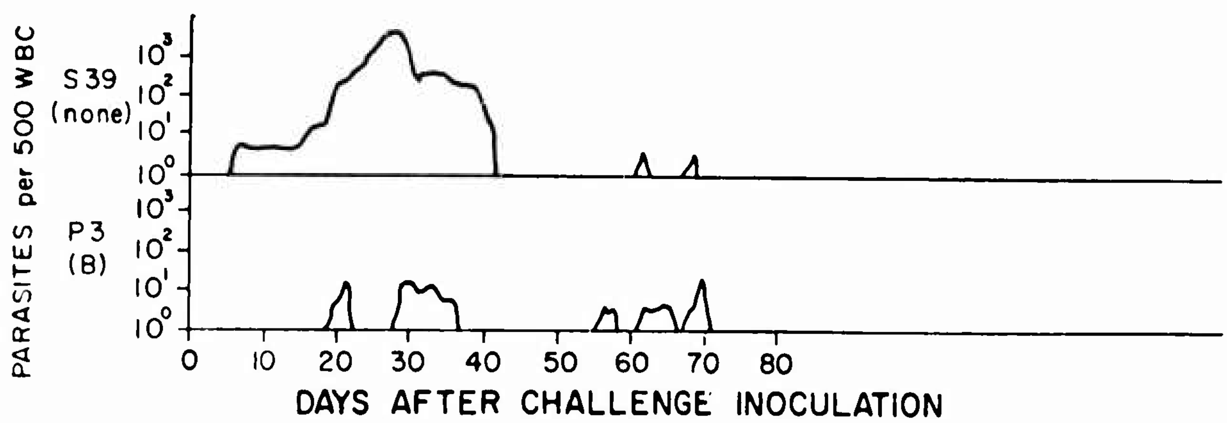


FIGURE 1. TEST I HOMOLOGOUS CHALLENGE WITH ISOLATE B

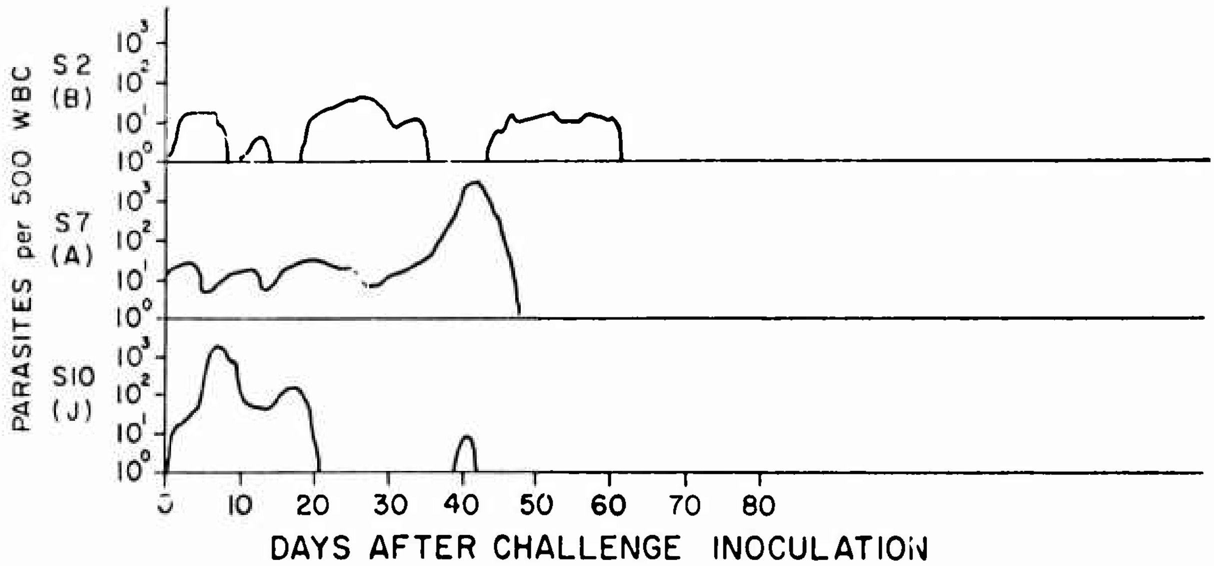


FIGURE 2. TEST II HOMOLOGOUS AND HETEROLOGOUS CHALLENGE WITH ISOLATE B

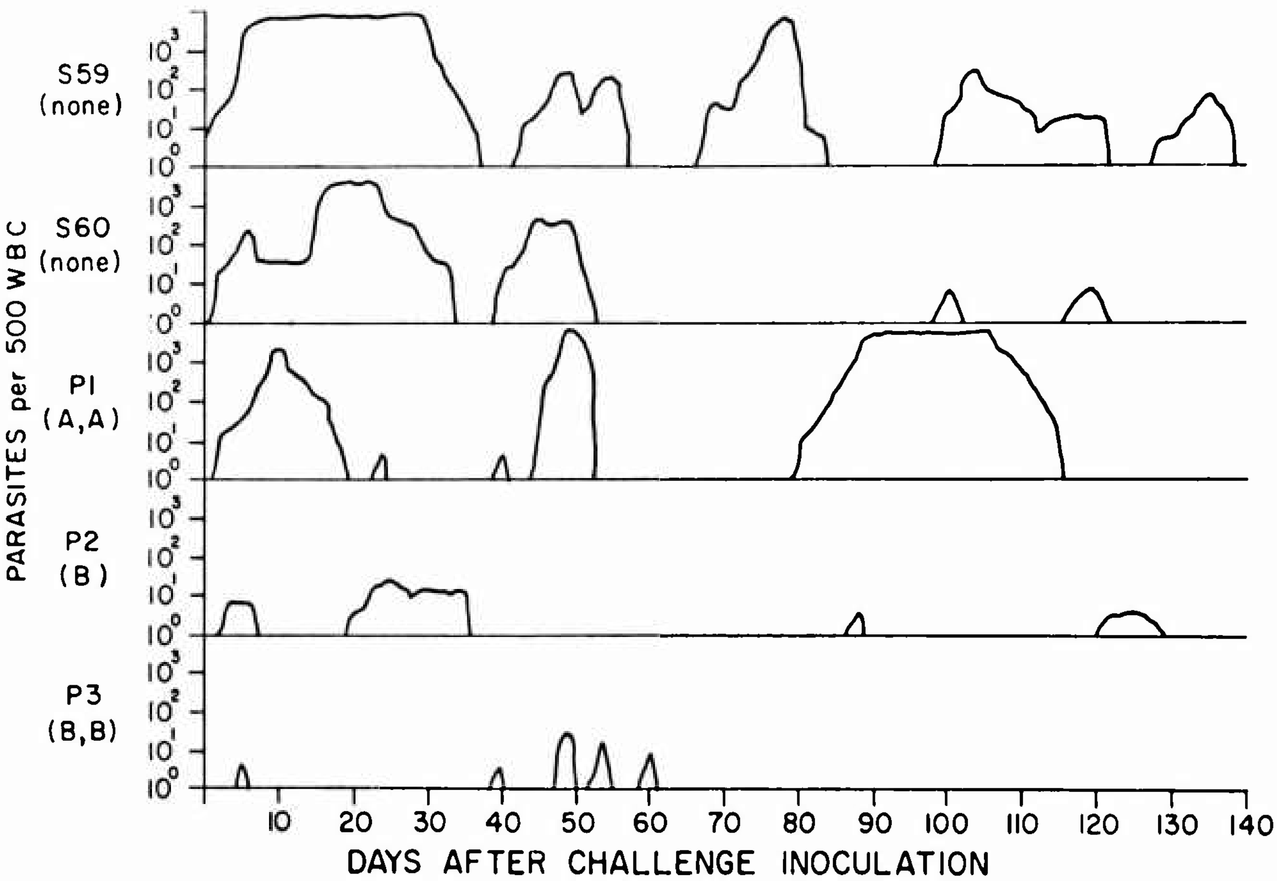


FIGURE 3. TEST III HOMOLOGOUS AND HETEROLOGOUS CHALLENGE WITH ISOLATE B

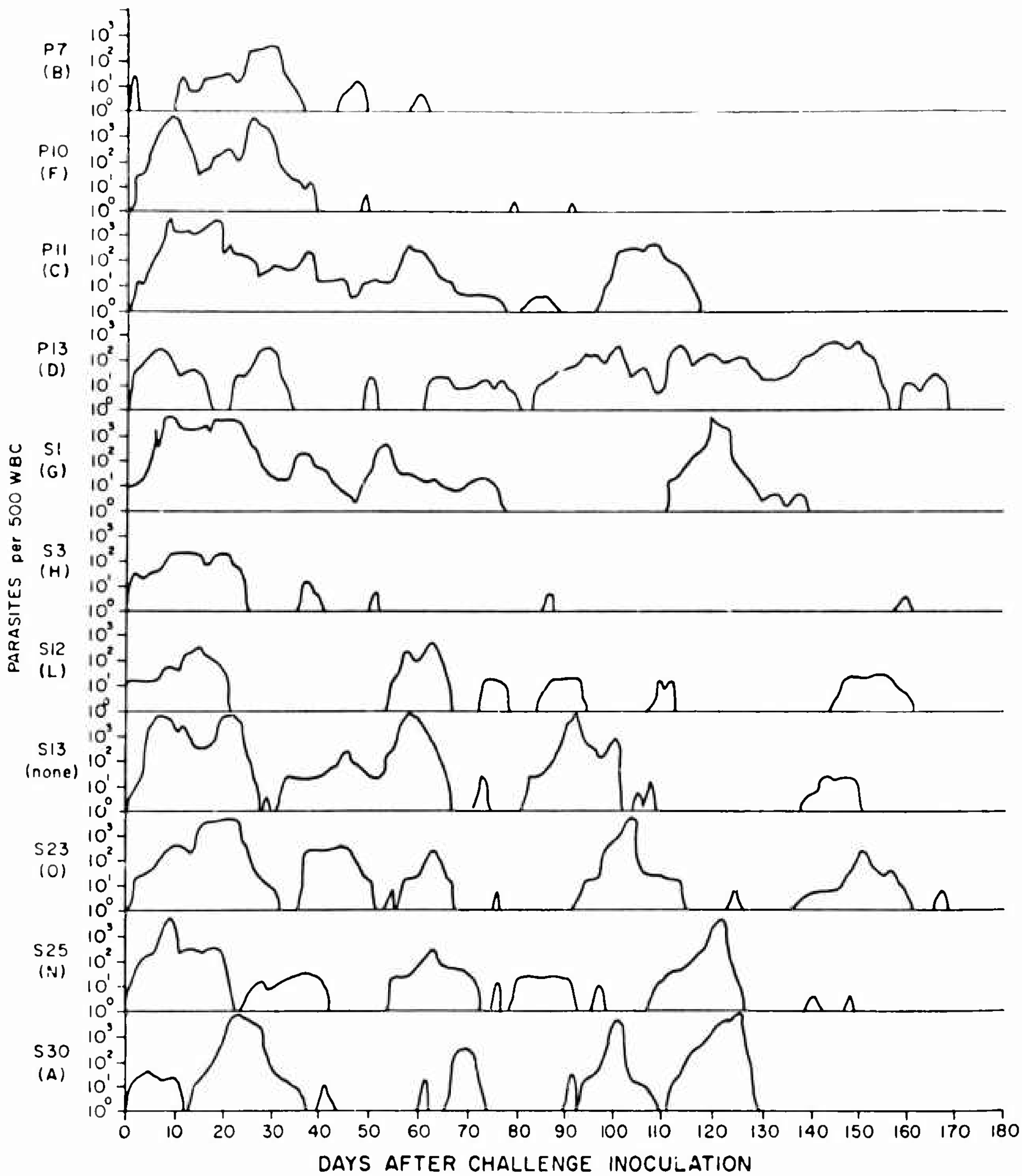


FIGURE 4 TEST IV HOMOLOGOUS AND HETEROLOGOUS CHALLENGE WITH ISOLATE B

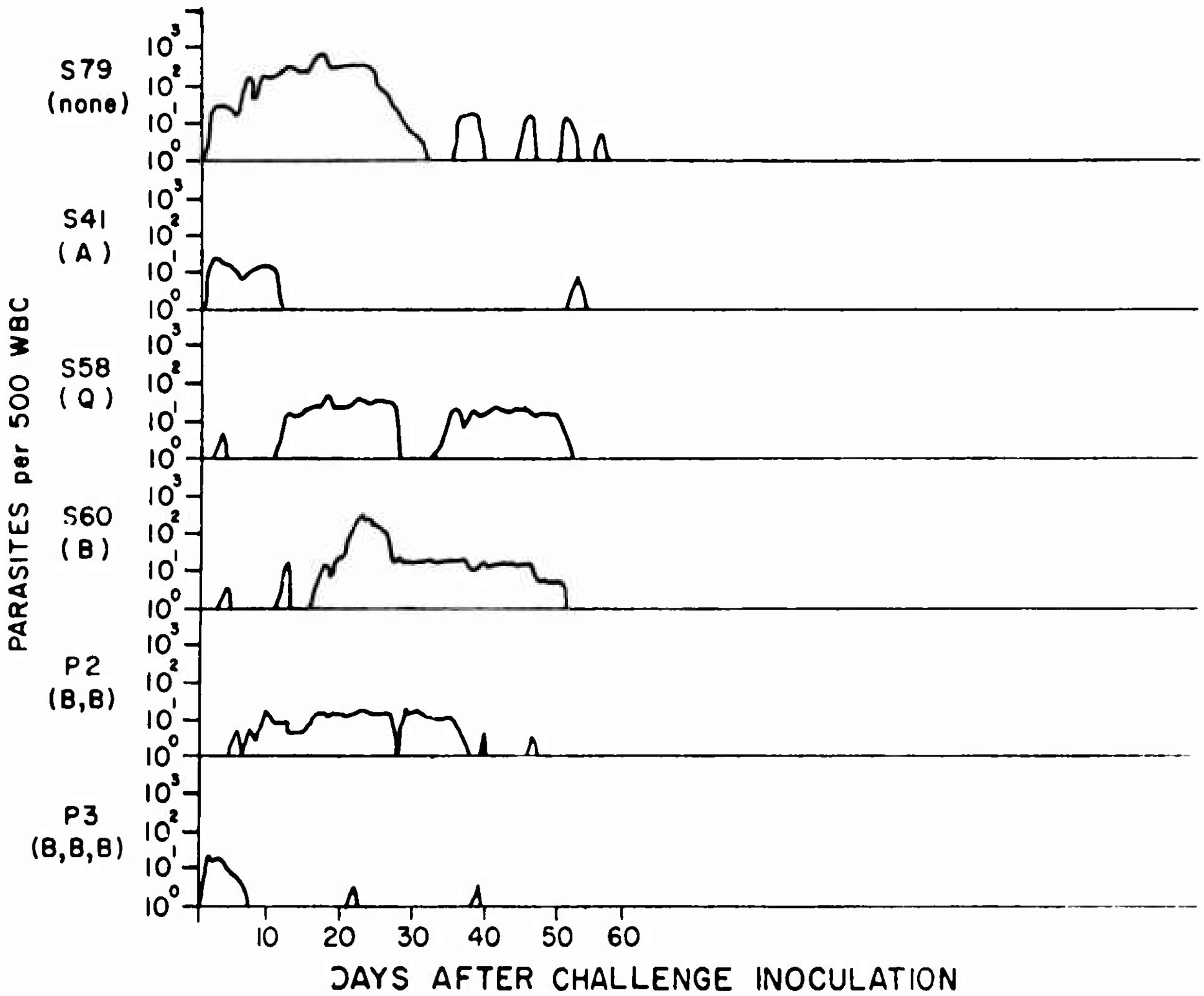


FIGURE 5. TEST V HOMOLOGOUS AND HETEROLOGOUS CHALLENGE WITH ISOLATE A

Subtitle: Comparative Studies in the Pathology and Host Physiology of Malarias: Gibbon Malaria.

Investigators: Louis H. Miller, Robert S. Desowitz, Vithune Yuthasastrkosol, Richard D. Buchanan, Barnyen Permpanich

Four plasmodia have been described as natural infections of S.E. Asian gibbons: Plasmodium hylobati (Rhodain, 1941), P. youngi (Eyles, et al 1964), P. eylesi (Warren et al, 1965) and P. jefferyi (Warren et al 1966). In addition, Hylobates lar may serve as an experimental host for P. falciparum (Ward et al; 1965; Ward and Cadigan, 1966). Virtually nothing is known of the host pathophysiology in these infections. Eyles et al. (1964) noted that gibbons infected with P. youngi showed a clinical illness fever, anaemia and lethargy.

The species of plasmodium employed in this present study cannot be given with certainty. We have observed forms corresponding to all four species occurring in any individual during the course of its infection. There may be some confusion in the taxonomy of gibbon plasmodia and an account of the morphology of the Thai isolate employed in this study will appear elsewhere. Despite this deficiency, it is felt that the host response of the gibbon is sufficiently unique to warrant this present paper.

METHODS

Adult Hylobates lar of Thai origin were used in these experiments. The principles of animal care as promulgated by the National Society for Medical Research were observed. Prior to the experiment, blood films were taken from each animal and only when negative for malaria parasites was the gibbon used. All animals were then given a full course of 4 amino and 8 amino-quinolines and experimentally infected not earlier than 3 months after completion of the chemotherapeutic regimen. Splenectomy was performed between one and ten months before experimental infection.

The plan of study and methods followed that for the P. coatneyi malaria (Desowitz et al, 1967). Five splenectomized (S2, S4, S14, S28, and P14) and one intact gibbons (P16) were infected by intravenous or intraperitoneal inoculation of parasitized blood. The inoculum ranged from $6.0X10^{6}$ to $5.5X10^{8}$ parasites. The plasmodium employed in this study was isolated from a naturally infected H. lar from Tak, Northwest Thailand. Seven ml. of blood was obtained weekly for haematology and serum chemistries (transaminases, alkaline phosphatase, direct and total bilirubin, blood urea nitrogen, and creatinine). Temperature and blood films for parasite density were taken daily. Bone marrow aspirations and biopsies of liver and kidney were made on selected animals.

Two control animals were maintained and haematology and blood chemistries obtained in the same manner as for infected animals.

RESULTS

Controls. Tables I and II show the weekly values for two uninfected gibbons over a period of 56 and 69 days. Table III summarizes the preinfection values for all experimental animals. While there was the expected variation of weekly haematologic values there was no indication of anaemia produced by the successive bleedings. Variations serum chemistries in individual animals over a period of time and between animals were of similar magnitude to those previously found for normal rhesus monkeys (Desowitz et al, 1967).

Infected gibbons.

The course of parasitaemia and host response were similar in all gibbons studied. Fig. 1 shows the parasitaemia, haematology and blood chemistries for one typical infection while fig. 2 presents the parasitaemia, haematocrit and serum cholesterol for all experimental animals. It will be seen from these figures that the outstanding features of this infection were a high unrelenting parasitaemia, severe persistent anaemia and hypocholesterolaemia.

There was a close relationship between parasitaemia and anaemia. This was further exemplified in S14, a gibbon given two courses of chloroquine treatment. At each reduction of parasitaemia there was a rapid increase in haemoglobin and haematocrit. The one exception to this pattern was P16, an intact gibbon. This animal recovered from the anaemia despite the continuing parasitaemia of a similar magnitude as that in splenectomized gibbons,

The continuously high indirect bilirubinaemia (0.5-1.8 my per cent) reflected the haemolytic process. There was no indication of servere bone marrow depression as an added factor in the etiology of the anaemai. The marrow showed erythroid hyperplasia with an M:E ratio of 1:2 rather than the normal ratio of 2:1. There was a reticulocytosis that occasionally attained 40 per cent and numerous nucleated red blood cells appeared in the peripheral blood. There was no depression of the white blood cell count.

Despite the intense, persistent anaemia most animals showed no overt signs of illness. They continued to eat and were not lethargic. S4, the only fatal infection encountered, appeared well until it sustained a parasitaemic recrudescence with rose from approximately 100 parasites per 50 thin film fields on the 153rd day to 600 parasites on the 158th day. This caused a further drop in the haematocrit, from 14 to 5 per cent, and the animal died.

The marked hypocholesterolaemia appeared to be associated with the anaemia. As will be noted from fig. 2 the rapid fall in cholesterol closely paralleled the progression of anaemia and returned toward normal levels only when the anaemia improved, such as in P16 and S14.

Little other serum chemistry abnormality was evident at any stage of the infection. There was no indication of hepatic abnormality. The transaminases showed no elevation and were remarkably constant during the entire period of observation in all the animals as exemplified in fig. 1. Furthermore, the two tests for liver function, direct bilirubin and alkaline phosphatase, were not elevated. Liver biopsies obtained on S4 (85th day of infection) and P14 (135th day) showed no features suggestive of functional alteration. The Kuppfer cells contained large masses of malarial pigment. The hepatic cell cytoplasm in S4 was irregular with clumping and vacuolization, more prominent near the central vein. Renal abnormalities were also absent as indicated by normal BUN and creatinine values. There was, in fact, a tendency for BUN to be lowered as the anaemia progressed. Renal biopsy on P14 showed no histopathology indicative of significant of functional alterations. There was moderate swelling of the glomerular endotherlium.

As in P. coatneyi infections, there was a trend toward lowered alkaline phosphatase levels during the infections. In some gibbons, such as S2 in which the alkaline phosphatase fell from 23 to 7 units, this was striking.

Discussion

Comparison of the pathophysiology of P. coatneyi in the rhesus monkey and gibbon malarias brings into focus two distinct pathologic conditions; acute P. coatneyi malaria, characterized by anaemia plus a "toxic" element which manifests itself in hepato- renal pathology and gibbon malaria in which there was an intense anaemia without any indication of other pathologic alterations. The parasitaemias in the splenectomized gibbons were at least as high as those in the acutely ill monkeys with P. coatneyi.

Furthermore, these high parasitaemias persisted for much longer periods than in the P. coatneyi infected rhesus. The ability of the gibbon to sust[illegible]in the burden of profound anaemia for long periods of time without overt signs of illness or secondary tissue pathology is truly remarkable. The underlying mechanism responsible for the development of organ lesions in malaria is not known with certainty. Maegraith (1948) has proposed that the combined effects of anoxaemia due to anaemia and haemodynamic circulatory changes induce the tissue pathology. With regards to the genesis of circulatory disturbances and consequent histotoxic effect he states, "I think changes in the permeability of the vascular endothelium are of supreme importance". Certainly anaemia per se, as indicated by this present study, did not produce observable organ pathology. It may be relevant that we have not been able to demonstrate in gibbon malaria the serum vascular permeability factor present in P. inui and P. coatneyi infections (Desowitz and Pavanand, 1967). However, the study is still in its early stages and this observation requires further confirmation. Fundamentally, we are again confronted with the old problem of virule[illegible] in host-parasite relationships. Is P. coatneyi more virulent than gibbon malaria, i.e., are there inherent [illegible]ences in the pathogenic potential between parasite species; or is the difference attributable to [illegible]ilarity of host response in gibbon and rhesus? Undoubtably both factors contribute to the comp[illegible]on of disease. Virulence might be defined as an expression of interacting parasite and host [illegible] Virtually nothing in known of the metabolism of the metabolism of gibbon or other primate pla[illegible] the meagre data available cannot, as yet, be rationally applied to an understanding of disease [illegible]on.

The most striking chemical change induced by gibbon malaria was a rapid and persistent fall in serum cholesterol. Hypocholesterolaemia has been observed in human malaria (Crespin and Zaky, 1919; Fairley and Bromfield;1933 McQuarrie and Stoesser, 1932; Kehar, Kopp and Solomon, 1943) in chronic P. knowlesi infections (Krishnan. et al, 1936: Kehar, 1937), and in P. coatneyi malaria (Desowtiz et al, 1967). The reduction of serum cholesterol is probably common to many infectious diseases. McQuarrie and Stoesser (1932) noted a definite fall in cholesterol during pneumonia, empyema, tosilitis and otitis media.

At present we can only speculate as to the cause of hypocholesterolaemia but serveral possible explanations can be offerred. These are; (1) dretary deficiency, (2) depletion from utilization by parasites and/or reticulocytes and reticuloendothelial elements, (3) impaired synthesis and (4) enhanced catabolism. The precipitous full in the cholesterol level would tend to obviate a dietary etiology since a reduced food intake does not appreciably alter serum cholesterol (Keys et al., 1950).

Depletion of cholesterol from incorporation into parasites or reticulocytes is a possibility although it would seem to be more of a contributory factor than a primary one. Morrison and Jeskey (1947) have shown that almost 30 per cent of the dry weight of P. knowlesi is material, predominantly cholesterol. More recently however, Wallace et al. (1965) found sterols in P. lophurae and P. berghei to be in a lower proportion than the phospholipids. Because of the similarity in cholesterol concentrations in P. knowlesi and host blood Williamson and Ginger (1965) postulated a direct uptake of sterols by the parasite from host blood. Reticulocytes may also play a role since these cells contain relatively large amounts of cholesterol, two to three ti[illegible]es that of mature erythrocytes (Raderecht et al., 1960). It has been clearly demonstrated that in gibbon malaria the hypocholesterolaemia is coincidental with the anaemia and reticulocytosis, but the nature of this association has not been elucidated. A search of the literature for other investigations relating to cholesterol level in haemolytic anaemias has not been productive. The increased activity and hyperplasia of reticuloendothelial elements during malaria is well known. Riggi and Di Luzio (1962) have shown that the stimulated reticuloendothelial system caused a reduction in hepatic and plasma ester and total cholesterol. Thus, this factor also could contribute to the hypcholesterolaemia of malaria.

We have no direct information as yet pertinent to the third possibility, decreased synthesis. Impairment of cholesterol synthesis is known to occur in diseases affecting the liver parenchyma but there was no biopsy or serum chemistry evidence of this in the infected gibbon. Moreover, in the acutely infected P. coatneyi rhesus with centrilobular necrosis the decrease in serum cholesterol was less in the than gibbon malaria. In this present investigation only total cholesterol was analyzed. Determination of esterified and free

cholesterol ratios should help clarify the question whether the liver is responsible for the hypocholesterolaemia. The fourth hypothesis, increased cholesterol catabolism, also remains to be investigated.

The hypocholesterolaemia itself may contribute to the etiology of the anaemia. Murphy (1962) has shown that cholesterol-depleted erythrocytes have an increased osmotic fragility. The cholesterol in the mature erythrocyte is in dynamic equilibrium with the serum nonesterified cholesterol (London and Schwarz, 1953) and presumably a state of hypocholesterolaemia would lead to a depleted erythrocyte.

Summary

1) Five splenectomized and one intact H. lar were inoculated with a plasmodium isolated from a naturally infected gibbon from Northwest Thailand.

2) The pathophysiology of the infections was studied in terms of pararitaemia, haematology blood chemistry, and histology.

3) The outstanding features of this infection were a high, unrelenting parasitaemia, severe persistent anaemia and hypocholesterolaemia. The hypocholesterolaemia coincided with the anaemic state.

Acknowledgements: We would like to thank LTC D. Snyder, MC and the clinical laboratory, Clinical Research Center, for performing the biochemical determinations and SFC Gene Clary for the haematology determinations. Animal care was under the supervision of CPT William E. Vick, VC.

TABLE I Haematology and blood chemistries of serial samples from control gibbon S73

Days	Haematology					Blood Chemistry							
	WBC /mm^3	RBC x10^6 / mm^3	Haemo globin gm%	Haemato crit %	Reticu-locytes %	Bilirubin mg%		SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha-tase Sigma Units	BUN mg%	Creati-nine mg%	Choles-terol mg%
						Direct	Total						
0	9,600	5.62	11.2	37	0.6								
6	5,400	5.88	12.1	38	0.4	0	0	35	21	22.0	11.2	0.9	113
13	6,400	5.36	9.9	33	0.8	0	0	25	23	29.2	11.7	0.8	112
20	4,700	6.52	10.3	35	0.9	0.05	0.2	22	18	22.0	14.2	1.0	111
27	6,100	5.40	9.5	31	0.4	0	0.4	31	16	22.0	5.9	0.7	
35	6,900	5.45	10.8	33	0.3	0	0	19	18	23.6	13.7	0.9	89
41	5,400	6.54	11.0	37	0.1	0.1	0.2	20	19	28.8	10.4	0.9	112
48	5,500	6.30	10.4	36	1.1	.05	0.3	26	25	26.8	18.3	1.0	113
63	5,000	5.90	12.2	41	1.1	0	0.1	22	17	30.0	12.1	0.8	129
69	6,200	6.40	13.8	36	0.2	0	0	22	17		13.8		
Range	4,700–9,600	5.36–6.54	9.5–13.8	31–41	0.1–1.1	0–0.1	0–0.4	19–35	16–25	22.0–30.0	5.9–18.3	0.7–1.0	89–129

TABLE II - Haematology and blood chemistries of serial samples from control gibbon S62

Days	Haematology					Blood Chemistry							
	WBC /mm³	RBC x10⁶ / mm³	Haemo-globin gm%	Haemat-ocrit %	Retic-ulocytes	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Unite	SGPT S.F. Unite	Alkaline Phosphatase Sigma Units	BUN mg%	Creatinine mg%	Choles-terol mg%
0	6,100	6.21	11.0	38	0.4								
7	4,600	6.09	10.5	38	0.2	0.1	0.1	43	44	26.6	13.7	1.5	186
14	6,100	5.87	10.6	39	0.5	0.3	0.2	46	35	19.0	11.4	1.1	170
21	7,800	6.90	11.3	41	0.4	0	0	52	41	14.6	11.5	0.7	188
28	6,300	6.37	11.0	38	0.2	0	0.1	48	32	25.0	10.0	0.9	170
35	9,900	6.20	11.0	38	0.7	0.05	0.1			16.0	12.3	0.8	142
42	6,600	5.79	10.8	36	0.4	0	0.1	40	36	16.0	14.9	0.8	154
49	8,000	6.23	11.2	37	0.4	0.05	0.1	39	26	19.8	11.2	0.8	138
56	5,600	6.32	10.1	39	0.8	0.1	0.4			14.8	12.9	1.1	150
Range	4,600–9,900	5.79–6.90	10.1–11.8	36–41	0.2–0.8	0–0.2	0–0.4	39–52	26–44	14.6–26.6	10.0–14.9	0.7–1.5	138–188

TABLE III – Summary of preinfection values of experimental gibbons

Gibbon	Haematology					Blood Chemistry							
	WBC /mm^3	RBC x10^6 / mm^3	Haemo globin gm %	Haemato crit %	Retic ulocytes	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha. tase Sigma Units	BUN mg%	Creatinine mg%	Cholesterol mg%
	8,800	6.12	13.4	45	0.4	0.05	0.2	30	26	23.2	6.6	1.1	154
	13,100	5.73	12.7	44	0.4	0	0.1	34	31	19.0	9.8	1.1	155
S2	11,700	7.14	13.1	44	0.8	0.05	0.1	21	20	23.6	10.2	1.0	160
S9	7,800	4.49	11.8	39	0.9	0.05	0.1	27	26	19.0	2.7	0.6	111
S4	6,500	5.71	11.4	38		0.1	0.5	38	37	15.0	19.7	0.7	130
S65	4,500	6.50	10.1	36	0.6	0	0.2	34	19	18.2	15.2	0.6	121
P14	7,200	5.73	12.7	41		0.05	0.4	36	28	18.0	17.0	1.2	148
S28	7,900	7.07	13.6	45			0.2	39	23	38.0	13.0		145
P16	5,900	6.60	13.4	43			0.4	43	28	14.0	11.0		174
S14	16,900	5.65	12.2	42	0.5	0.05	0.2	32	29	31.6	11.7	1.0	128
	16,100	5.82	12.1	41	0.9	0.1	0.1	31	26	27.2	14.2	0.9	130
	12,500	6.91	12.7	43	0.7	0.05	0.1	29	25	31.2	12.9	0.9	131
Range	4,600– 16,900	4.49– 7.14	10.1– 13.6	36–45	0.4– 0.9	0– 0.1	0.1– 0.5	21–43	19–37	14.0– 38.0	2.7– 19.7	0.6– 1.2	111–174

Fig. 1. Parasitaemia, haematology and blood chemistries during a typical course of infection in a splenectomized gibbon.

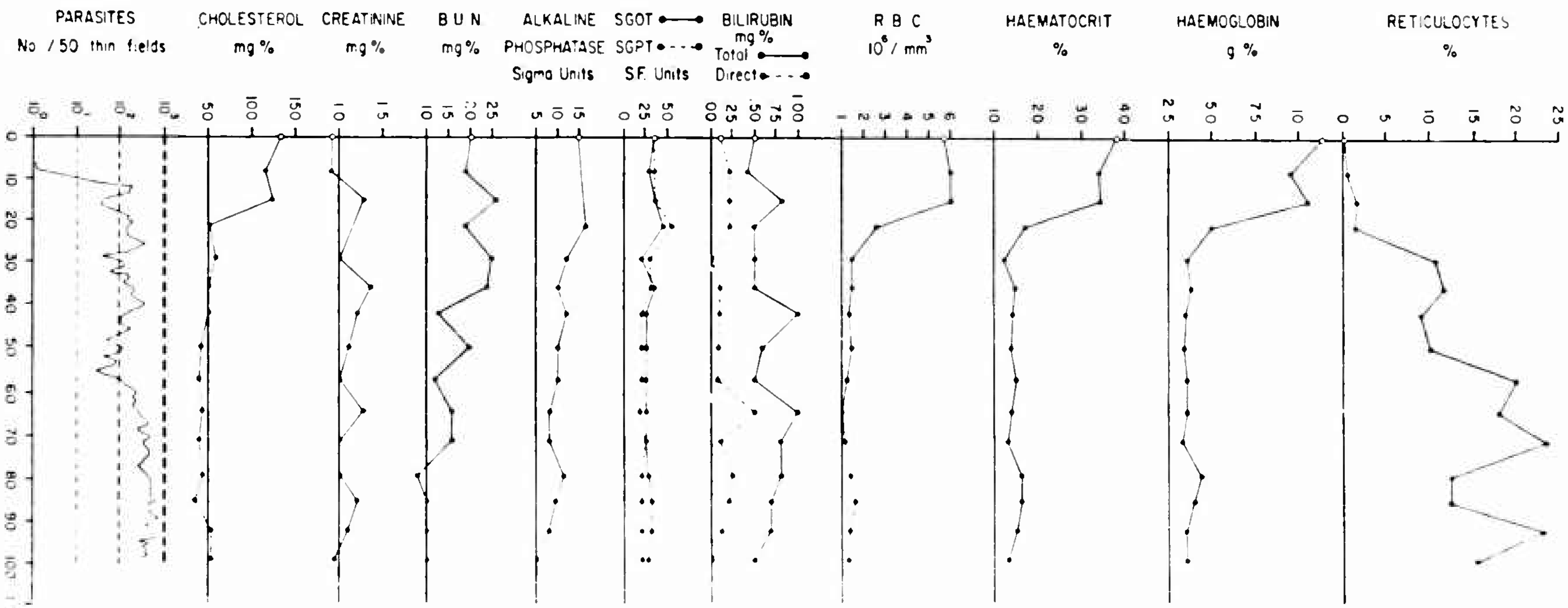
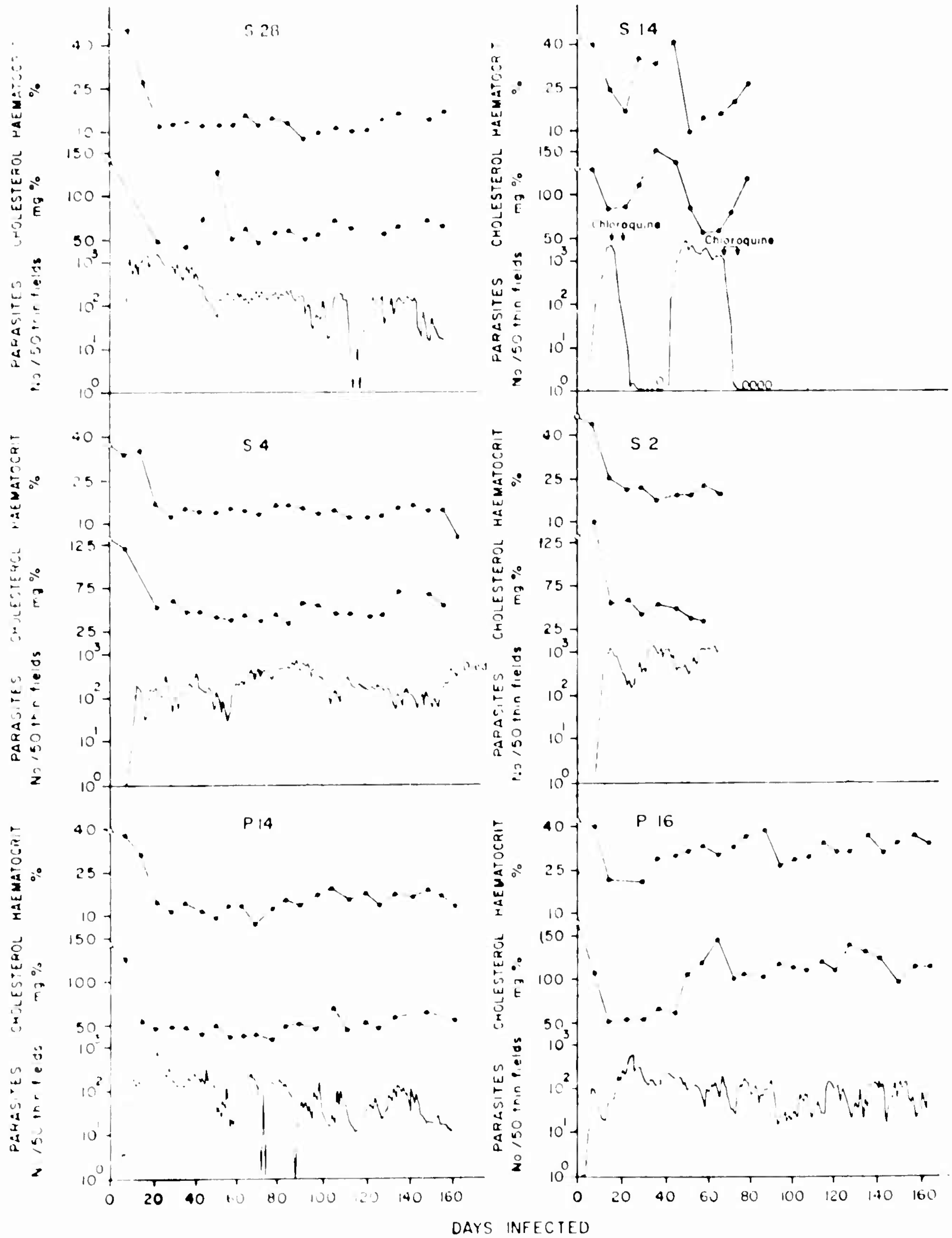


Fig. 2. Parasitaemias, haematocrits and cholesterol levels during the course of infections in all experimental gibbons.



Subtitle: Comparative Studies in the Pathology and Host Physiology of Malarias: Plasma Free Fatty Acids in Normal and Malarious Gibbons.

Investigators: Bernhardt W. Langer Jr., Robert S. Desowitz, Louis H. Miller, Duangduen Vacharaphorn

Introduction:

It has been observed that there was a decrease in serum cholesterol in malarious gibbons (Miller, L.H., Desowitz, R.S., Yuthasadtrkosol, V., Buchanan, R.D., and Permpanich, B. 1967. Comparative studies in the pathology and host physiology of malaria. II. Gibbon Malaria (To be submitted for publication). The question was raised as to whether this change represents a generalized lipid metabolism defect or was limited to cholesterol.

Objective:

The objective of this study was to examine the plasma free fatty acid level of normal, intact; normal, splenectomized; infected, intact; and infected, splenectomized gibbons in order to determine the effects of malaria infection on this parameter.

Methods:

Gibbons, normal or infected, intact or splenectomized, maintained in our animal colony were used throughout the study. Five ml. of blood was drawn into a heparinized syringe after an overnight fast (1700 hrs. to 0800 hrs.) and the plasma immediately analyzed for free fatty acids using the method of Kvam et al. (Kvam, D.C., Schmidt, J.G., Riggils, D.A., and Galls, D.G. 1964. Colorimetric micro-determination of plasma free fatty acids. J. Pharmaceut. Sci. 53:988).

Progress:

Twenty-seven gibbons were examined in this study, the results being shown in Table I. No significant differences were observed between any of the four groups studied. These data indicate that the gibbon malaria apparently does not affect the transport of free fatty acids and does not drastically alter the anabolic or catabolic metabolism of free fatty acids.

publication entitled "Comparative Studies in the Pathology and Host Physiology of Malaria. III. Plasma Free Fatty Acids In Normal and Malarious Gibbons (*Hylobates lar*). by B.W. Langer, Jr., R.S. Desowitz, L.H. Miller, and D. Vacharaphorn reporting the results of this study has been submitted for clearance.

Summary:

There is no significant differences in plasma free fatty acids between normal and malarious gibbons.

TABLE I

Plasma Free Fatty Acids in Normal and Malarious Gibbons.

Gibbon treatment	Ave. FFA level (umoles/ml)*	S.D.	Range	N
Normal, Intact	0.306	0.088	0.130–0.457	15
Normal, Splenectomized	0.397	0.099	0.263–0.484	6
Infected, Intact	0.374	0.026	0.357–0.391	2
Infected, Splenectomized	0.329	0.125	0.183–0.477	4

* Stearic acid was used as the standard in the analysis for FFA.

Subtitle: Comparative Studies in the Pathology and Host Physiology of Malarias: *Plasmodium coatneyi* malaria.

Investigators: Robert S. Desowitz, Louis H. Miller, Richard D. Buchanan, Vithume Yuthasastrkosol, Barnyen Permpanich

Because of the multiplicity of host-parasite relationships in the genus *Plasmodium* a full understanding of host pathology necessitates a comparative approach. Not only do the different species of *Plasmodium* produce a variety of diseases but also the type of infection any single species may induce can vary within its host range. A familiar example of this is the benign course of *P. knowlesi* in *Macaca irus* as compared to the fatal infection produced in *M. mulatta*.

Although the work of Maegraith and his colleagues on *P. knowlesi* over the past two decades has greatly contributed to the understanding of malarial pathophysiology, many other malarias have not been similarly examined. In view of this hiatus it is the aim of these present studies to describe and compare the disease produced by various malarias in different hosts. This initial paper is concerned with a primate malaria of S.E. Asia, *P. coatneyi* (Eyles et al. 1962), in the rhesus monkey. This parasite shares many characters in common with *P. falciparum*. With the exception of the gametocytes, the morphology is similar and it exhibits a tertian periodicity with deep vascular schizogony (Eyles et al. 1962). There is also evidence that *P. coatneyi* is antigenically related to *P. falciparum* (Stein and Desowitz, 1964; Desowitz, Saave, and Stein, 1966). For these reasons *P. coatneyi* is of special interest as a possible model for human malignant tertian malaria.

Methods

Adult rhesus monkeys from Northern India (*M. mulatta mulatta*) and Thailand (*M. mulatta siamica*) were used in these experiments. Repeated blood examinations gave no evidence of malaria and tuberculin skin tests showed all to be free of tuberculosis. Some animals were splenectomized prior to experimental infection. The monkeys were infected by intravenous or intraperitoneal inoculation of parasitized blood. The description of each animal will be given under Results. Four animals were maintained as uninfected controls.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

The course of infection was studied in terms of parasitaemia, temperature, haematologic values, various blood chemistries, urinalysis, and tissue pathology. Haematology and blood chemistries were obtained on all animals prior to infection. The methods employed were as follows:

1. Parasitaemia. Blood films were obtained each morning. The parasitaemia was assessed by counting the total number of asexual forms in fifty high-power oil immersion fields of the Giemsa stained thin blood film.

2. Temperature. Rectal temperatures were taken each morning.

3. Haematology. One ml. of blood was drawn weekly into a heparinized syringe. In some animals blood was obtained more frequently as to be described in the results. Haematocrit, haemoglobin, red blood cell count, reticulocyte count, and white blood cell count and differential were performed. Bone marrow aspirations were obtained from selected animals. A bone marrow needle was inserted into the greater trochanter and the marrow smear stained with Giemsa-Wright.

4. Blood chemistries. At the time the blood was drawn for haematology another seven ml. was obtained for blood chemistries. This was allowed to clot, the serum obtained and refrigerated at 5°C. Transaminases and alkaline phosphatase determinations were carried out that same day. The remainder of the sample was stored at -70°C and the other analyses made within three days.

Serum glutamic-oxaloacetic transaminase (SGOT), serum glutamicpyruvic transaminase (SGPT) and alkaline phosphatase determinations were performed with the Sigma Test Kit (Sigma Chemical Co., St. Louis, Mo.). Direct and total bilirubins were performed by the method of Malloy and Evelyn (1937). Cholesterol analysis was made by the method of Zurkowski (1964). Creatinine values were obtained by the Technicon Autoanalyzer. Blood urea nitrogen (BUN) was determined by the Hyland UN Test Kit method (Hyland Laboratory, Los Angeles, Calif.).

5. Urinalysis. Urine was collected in a metabolic cage, daily for some animals and at varying intervals for others. It was analyzed for blood and protein by the Hema-combistex (Ames Co. Inc., Elkhart, Ind.). The centrifuged sediment was examined for casts, red blood cells and white blood cells.

6. Tissue pathology. Biopsies of liver and kidney were carried out in selected monkeys. Liver biops was made with a Menghini needle and kidney biopsy with the Franklin modification of the Vim-Silverman needle. The tissue was fixed in 10% neutral buffered formalin. In fatal infections complete postmortem pathological examination was carried out.

7. Controls. Four control animals had eight ml. of blood drawn weekly for haematology and blood chemistry. This was the same amount as obtained from the infected animals.

Results

Control monkeys

Tables I-IV show the range of values obtained from four control monkeys which had been bled weekly over periods ranging from 55 to 130 days. While the data indicate a variation in haematologic values, in no animal was there a progressive fall in erythrocytes, haematocrit or haemoglobin; nor was there a reticulocytosis. Table V summarizes preinfection values for all experimental animals including some monkeys that were subsequently infected with species of malaria other than *P. coatneyi.* It will be seen that these monkeys, in comparison to normal humans, have a lower haematocrit and haemoglobin in relation to the red blood cell count. Bone marrows from two control animals, SP9 and MS37, showed a cellular distribution and myloid: erythroid (M:E) ratios of 1.7:1 and 2.3:1. The general appearance of the marrow seemed similar to that of the normal human.

Tables I to V show the variation that may occur in some blood chemistries in any single animal over a period of time and between individual animals. Direct and total bilirubin, cholesterol, BUN, creatinine and SGPT showed relatively little variation. However, considerable differences were observed in SGOT values between individual animals. For example, the SGOT for MS37 was 20 sigma Frankel (S-F units) units while that of KL13 was 73. A variation in alkaline phosphatese was noted between the Indian rhesus (KL series) and the Thai rhesus (SP and MS series), the former showing higher levels.

In the four control animals the rectal temperature varied between 100 and 103°F with the exception of a few days in SP9 when the temperature fell to a low of 98°F.

Infected monkeys

The course of infection and attendant alterations in haematology, blood chemistry, and pathology differed considerably from animal to animal. In some monkeys the infection pursued a fulminating, fatal course while in others there was a primary parasitaemia of varying intensity followed by chronicity. The type of disease appeared to be influenced by the strain of monkey (Thai or Indian rhesus) and prior splenectomy. In view of these many individual differences the history of each animal will be presented in detail.

KL13. (splenectomized Indian rhesus). The data obtained from this animal are shown in fig. 1. Parasites were present in the thick blood film on the second day after intravenous inoculation of $2x10^6$ parasites. There was a progressive increase in the parasitaemia with a peak of 2550 parasites per 50 thin film fields on the 9th day. The parasitaemia fell slightly to 1474 on the 10th day and remained at this level until the animal died during the night of the 11th day.

On the 6th day both transaminases rose significantly although there was no concurrent decrease in haematologic values. When the peak parasitaemia was attained on the 9th day, a reduction of all haematologic parameters was observed. Temperature rose to 105°F at this time. On the 10th day the animal was lethargic and lying on its side. By the terminal day there was a severe anemia (haematocrit 16%) with intense haemoglobinuria and proteinuria. The temperature had dropped to 97°F. The transaminases having returned to normal on the 9th day, rose again (SGOT 158 SGPT 86). Both BUN and creatinine were abnormally high only on the day of death. Serum cholesterol decreased from a preinfection value of 165 mg per cent to 115 mg per cent on the day of death. No significant change of white blood cell count occurred at any time.

A liver biopsy taken on the 8th day had occasional areas of early centrilobular necrosis. There was a heavy round cell infiltrate in the periportal areas. The Kupffer cells contained large amounts of pigment. At autopsy there was massive centrilobular necrosis of the liver with engorgement of the sinusoids.

The renal tubules were not strikingly altered. The glomerular tufts were infiltrated by mononuclear cells. The lung contained scattered foci of atelectasis. There were intravascular clots at many sites.

KL12, (splenectomized Indian rhesus). This animal sustained a rapid, fatal infection. Parasites were first detected on the 6th day after the intravenous inoculation of $3x10^6$ parasites and reached a peak of 464 parasites per 50 thin blood-film fields on the 30th day. The animal died during the night of the 11th day. While the temperature was not elevated during any phase of the parasitaemia it dropped terminally to 98°F. There was an intense haemoglobinuria before death. No haematology or blood chemistries were taken for this animal.

Liver biopsy taken before infection showed normal morphology. The liver lesion (fig. 2) at autopsy was essentially the same as that of KL13 but the amount of centrilobular necrosis was greater. Kidney changes (fig. 3) were also more marked than in KL13. There was extensive hyaline droplet degeneration in the renal epithelium of the proximal convoluted tubules. Many haemoglobin casts were present in the collecting ducts. The presence of a large amount of protein rich fluid in Bowman's space would suggest a leakage of protein through the glomerular basement membrane.

KL3. (splenectomized Indian rhesus). The animal received an intravenous inoculation of $4x10^6$ parasites. It sustained a severe infection which produced overt signs of illness (fig. 4). Unlike KL12 and KL13 this monkey survived, the infection ultimately becoming chronic. The acute primary attack from the 6th to 14th days was studied in detail. A peak parasitaemia of 1740 per 50 thin film fields occurred at 1500 hours on the 9th day. It is of interest to note how rapidly parasite numbers may build up in the peripheral blood since the parasitaemia at 1000 hours on that day was only 157. Similar flucuations in parasitaemias were also noted when counts were made twice daily on the 8th and 10th days.

On the 6th day, by which time the parasitaemia was 46 per 50 thin film fields, the haematocrit had fallen from 37 to 32 per cent and nucleated red blood cells were present in the peripheral blood. However by the 8th day when a second parasitaemic peak had attained a density of 1032, all haematologic values taken at 1000 and 1500 hours increased, indicating a haemoconcentration. Throughout this day the monkey appeared extremely ill; it was lying on its side and unable to raise its head. The serum sample taken at 1000 hours showed a rise in BUN and creatinine and by 1500 hours the SGOT was elevated. On day 9 a parasitaemic crisis had occurred with a fall in parasite density to 157. The haemogram had declined to a level slightly below that prior to the haemoconcentration. There was a leucopenia of 3400 wbc/mm^3 . The animal's status had improved and it was able to walk about in the cage. BUN, creatinine and transaminases remained elevated. There was a precipitous decline in blood values on the 10th day and haemoglobinuria appeared for the first time (urine was collected continuously throughout the period of study). That the animal was beginning to respond to its anaemia even at this time was indicated by a reticulocytosis of 6% and the presence of 42 normoblasts per 100 white blood cells. The animal again

appeared ill and was lethargic. The BUN attained a maximum level of 51 mg. Per cent. By the 11th day the haematocrit showed no further decrease. The haemoglobinuria had disappeared and the animal was again walking about its cage. BUN and transaminases had fallen, but over the next three days the transaminases rose again. The creatinine remained normal and the BUN continued to be slightly elevated. On the 14th day there was a marked reticulocytosis of 21% and 169 normoblasts per 100 white blood cells.

Throughout the entire two week period of the acute phase the only abnormal temperature noted was on the 12th day when it fell to 97 °F. The akaline phosphatase declined from a preinfection level of 17.6 sigma units to 6.4 on the 14th day. There was also some decrease in cholesterol over this period. Other than the transient haemoglobinuria no other abnormalities were observed in the urine during the acute or chronic infection.

MS2. (splenectomized Thai rhesus). The monkey received an intraperitoneal inoculation of $5x10^6$ parasites. The primary attack was characterized by a high, unrelenting parasitaemia for 7 days followed by a second rise in parasitaemia with its peak on the 33rd day (fig. 5). Thereafter, the infection became chronic with a low-grade parasitaemia of tertian periodicity. The onset of anaemia at the 7th day occurred before the peak parasitaemia. During the period of the primary parasitaemic attack the transaminases, BUN, and creatinine rose. These blood chemistries returned to normal by the 30th day except the elevated SGOT which persisted for approximately 4 more weeks. Despite the abnormal SGOT a liver biopsy taken on the 23rd day was normal except for a heavy deposit of pigment in the Kupffer cells.

A peak of 20 per cent reticulocytes occurred on the 20th day and returned to a pre-infection level as the anemia disappeared with the onset of chronicity. Concurrent with the maximal reticulocytosis the highest number of normoblasts, 31,000 per mm^3, was present. The bone marrow on the 30th day showed erythroid hyperplasia with a M:E ratio of 1:3. There was a persistent leucocytosis that began during the acute infection and continued for the five month observation period. During the early chronic period the monocyte count rose to 45 per 100 WBC. No abnormal blood chemistries were evident during the chronic period.

MS18. (splenectomized Thai rhesus). The animal received an intraperitoneal inoculation of $5x10^6$ parasites. For the first 60 days a parasitaemia of 200 to 500 parasites per 50 thin film fields appeared with regular tertian periodicity (fig. 6). After day 75 the parasitaemia was of a chronic, low grade nature. A progressive anaemia developed during the first two weeks of infection and persisted until approximately day 75. There was a reticulocyte response of fluctuating intensity with a maximum of 9 per cent during the period of anaemia. With the onset of the low-grade chronic parasitaemia the haematologic values returned to normal. Normoblasts were first seen on day 21 and were present throughout chronicity with a maximum of 14/100 WBC. Bone marrow specimens taken on the 88th and 115th days were hyperplastic with M:E ratios of 1:2 and 1:3.5, respectively. There was no abnormality of white blood cell count at any time. Blood chemistries remained normal throughout the observation period with the exception of a rise in alkaline phosphatase. Urines on 63rd and 73rd days were normal.

A liver biopsy obtained on the 17th day showed increased activity of reticuloendothelial elements, much finely divided malarial pigment in the Kupffer cells and marked granularity and basophilia of the parenchymal cytoplasm.

KL1. (intact Indian rhesus). This animal developed a moderate primary parasitaemia which decreased progressively to a persistent low grade infection (fig. 7). It became severely anaemic by the 12th day followed by a gradual recovery towards normal. The anaemia was accompanied by a reticulocytosis, the presence of normoblasts in the peripheral blood, and increased unconjugated serum bilirubin. There was no significant alteration in the white blood cell count. The BUN rose to 32 mg. per cent on the 12th day and was normal thereafter. There was no coincidental rise in creatinine. The trasaminases and alkaline phosphatase did not, at any time, alter markedly. Cholesterol was decreased from a preinfection level of 162 mg. Per cent to 93 mg. per cent on the 17th day and returned to normal over the next two weeks. A liver biopsy carried out on the 26th day was normal except for pigment in the Kupffer cells.

KL2. (Intact Indian rhesus). This animal was inoculated intravenously with 7×10^7 parasites. The course of infection was characterized by a primary parasitaemia which became periodic after the 20th day (fig. 8). A peak parasitaemia of 542 parasites per 50 thin film fields occurred on the 26th day. After the 38th day the parasitaemia was scanty. The haemogram indicated a marked anemia by the 12th day. At that time a bone marrow specimen sbowed erythroid hyperplasia with a M:E ratio of 1:2. Further evidence of bone marrow activity was a peak reticulocytosis of 18 per cent on the 18th day and 46 normoblasts per 100 WBC. There was no alteration in the white blood cells. Neither transaminases, direct bilirubin, BUN nor creatinine on the 4th, 7th, and 11th days showed any alteration from baseline normal values. During this acute phase the alkaline phosphatase fell from a baseline average of 17 sigma units to 4. It remained at this low level until the onset of chronicity at the 40th day. A liver biopsy on the twelfth day showed nuclear irregularity and intense cytoplasmic basophilia in the parenchymal cells, histologic evidence of increased liver cell activity. Kupffer cells contained finely divided pigment. The sole abnormality of the kidney biopsy was minimal swelling of the glomeruli.

During the period of the 20th to the 38th day, a time when the animal was subjected to repeated parasitaemic attacks of moderate severity, the anemia persisted. With the decline in parasitaemia there was a return of haematologic values towards normal. From the 40th to 61st days there was an elevation of unconjugated bilirubin, an occurrence that had not been found in other monkeys during the chronic phase. The elevated bilirubin is probably a result of haemolysis and it is of interest to note that there was also a concurrent reticulocytosis. The cause of this haemolysis during a time of low parasitaemia was not known. Other than the unconjugated bilirubin and alkaline phosphatase there were no changes in blood chemistries.

On 10 separate days the temperature rose above 104°F. During 69 days of daily preinfection temperatures the maximum recorded was 103°F. There was no relationship between fever and parasitaemic periodicity.

SP1. (intact Thai rhesus). This animal received an intravenous inoculation of 9×10^7 parasites. The infection was of a mild nature with a parasitaemia that never exceeded 100 parasites per 50 thin film fields. From the onset, there was a tertian pattern of parasitaemia. After the second week, the infection became chronic with parasite numbers of not more than 10 per 50 thin film fields. Relatively little anaemia was produced and this only during the second week of infection. At this time the haematocrit had fallen from 33 to 21 per cent, with a concomitant reticulocytosis of 5 per cent. No alteration in transaminases, alkaline phosphatase, BUN or creatinine were observed. Cholesterol decreased from 145 mg. per cent to 90 mg. per cent early in the infection and remained low for five weeks, after which it gradually returned to the preinfection level. Urines collected daily for the first month of infection contained no protein.

DISCUSSION

The models commonly employed for physiologic studies, P. berghei in mice and P. knowlesi in the rhesus monkey, generally produce fatal infections. However human malaria differs from these in that the severity of disease is highly variable. Similar to human malaria P. coatneyi produced a spectrum of disease, ranging from a fulminating fatal infection as in KL12 and 13 to a mild chronic course as in SP1. The intensity of disease for experimental purposes can be controlled by using splenectomized or intact rhesus.

In general, the pathologic effect as manifested by alterations in haematology, blood chemistry and tissue pathology was related to the degree of parasitaemia. Exceptions to this did occur. A relatively low parasitaemia, produced a fatal infection in KL12 and a severe anemia in KL1. However, the limitations in parasite enumeration must be taken into account. Marked hourly flucuations in parasite density were seen in KL3, and thus in many of our animals from which blood films were only taken daily the true peak parasitaemia may well have been missed. Furthermore since schizogeny occurs in the deep vasculature, enumeration from peripheral blood would not give a true account of the total parasitaemia.

The most consistent pathologic effect was anaemia. In most cases, onset and degree of anaemia paralleled the rise in parasite numbers of the primary attack. However in one animal, MS2, an anaemia was produced on the 7th day, a time when the parasitaemia was still low. In the three acutely infected animals, KL12, KL13, and KL3, the rapid onset of anaemia was accompanied by haemoglobinuria (lytic phase), a phenomenon shown to occur in P. knowlesi (Devakul and Maegraith, 1959).

In KL3 a definite haemoconcentration occurred early in the infection, prior to the lytic phase. This event was associated with marked signs of illness and biochemical change. Haemoconcentration with an associated shock-like syndrome has been described by Devakul and Maegraith (1959), Skirrow (1962) and Chongsuphajaisiddhi (1966) in P. knowlesi infections and by Kean and Taylor (1946) in P. falciparum. This may be caused by a decrease in plasma volume which in turn may be related to the vascular permeability increasing factor in the serum of malaria infected monkeys (Desowitz and Pavanand, 1967). A haemoconcentration phenomenon may be extremely important in contributing to the early pathogenesis of malaria but it will require further detailed study on haematology, plasma volume and red cell mass to elucidate its frequency and mechanism.

In non-fatal infections there was a vigorous host response to the anaemia as evidenced by reticulocytosis, nucleated red blood cells in the peripheral smear and erythroid hyperplasia of the marrow. In most animals the peak reticulocytosis occurred approximately one week after maximum anemia. The duration of anemia seemed to depend upon parasite numbers.

Liver pathology was evident in some animals, notably those which sustained an acute infection. In these, centrilobular necrosis of the liver was accompanied by transaminase elevations. Centrilobular necrosis has previously been described as a consequence of P. falciparum in man and P. knowlesi in the rhesus monkey (Rigdon and Stratman-Thomas, 1942; Andrews, 1948). It is possible that two events are involved in the process of liver pathology due to P. coatneyi. In two acutely infected monkeys studied in detail KL3 and KL13 there was an initial rise in transaminase with biopsy evidence of patchy centrilobular necrosis in one. This was followed by a decrease in transaminase levels and secondary elevation at or shortly after the lytic phase. The SGOT usually rose to higher levels than the SGPT which is in keeping with Maegraith's findings for P. knowlesi (1966). However, Sadun et al. (1966) found that the SGPT increment was greater than SGOT in chimpanzees infected with P. falciparum. This difference might be attributed to the host species studied.

Of the five moderately infected animals, only MS2 had an elevated transaminase but there was no histologic evidence of hepatic necrosis. In the other monkeys there was no transaminase elevation over the period of rapidly developing anaemia. Maegraith (1966) has raised the point that hemolysis might contribute to a transaminase elevation but we have no evidence for this in either these experiments or in gibbon malaria which is to be reported in a subsequent paper.

The two biochemical indicators of liver function employed in this study, direct bilirubin and alkaline phosphatase, showed no abnormal elevation in either acutely or moderately infected monkeys. In fact, there was a tendency for alkaline phosphatase level to fall. This is similar to the finding of Sadun et at (1965) for mice infected with P. berghei. At present, it is difficult to account for this decrease in alkaline phosphatase during malaria. It has been shown that this enzyme is high in growing animals and falls when growth is retarded (Gutman, 1959). It is possible that malaria inhibits the growth process and that the serum alkaline phosphatase level might reflect this.

Similar to hepatic changes, renal abnormalities were most frequently observed in the acutely infected animals. In both KL3 and KL13 the BUN and serum creatinine were abnormably high. In KL3 this occurred early, at the time of haemoconcentration and inital rise in transaminases, while in KL13 this increase was a terminal event. Despite the abnormal biochemistry, the kidney pathology of the autopsy specimen of KL13 showed remarkably little change. That severe histopathologic alteration of the kidney may occur was shown in KL12 where the picture was similar to that described for P. knowlesi of the rhesus (Rigdon and Stratman-Thomas 1942).

In the more moderate infections renal abnormalities were less consistently observed. MS2 exhibited an elevation of both creatinine and BUN, coinciding with a rise in transaminases. In KL1 only the BUN increased without any alteration in either creatinine or transaminases. The elevation of BUN when it occurs without coincidental rise in creatinine may be attributed to an increased metabolic production of urea. An abnormal serum creatinine without tissue pathology suggests a decreased renal blood flow. Evidence for renal blood flow abnormality has been presented by Chongsuphajaisiddhi (1966) for P. knowlesi and Sitprija et al (1967) for three patients with severe P. falciparum infections.

Transient decreases in cholesterol were seen in five of seven monkeys. The significance of this will be discussed in a subsequent paper when a comparison of cholesterol alterations in malarias will be made.

There was no consistent relationship between temperature elevations and parasitaemia or other manifestations of disease. The inconsistent finding of pyrexia in this disease and its virtual absence in P. knowlesi malaria (Menon and Nair, 1955) is in sharp contrast to the human host who, in this respect, would seem to be exquisitely sensitive to the parasite. The temperature fell late in the acute infections and this may be related to shock.

While it is obvious that there is no perfect model for human falciparum malaria we feel that P. coatneyi infection in the rhesus monkey approaches this ideal more closely than any other primate malaria studied hitherto. Not only is it morphologically and antigenically related but as has been shown in this paper the general pathogenic effects are, in many respects, similar to the human infection. However it is not our intention at this time to present a detailed comparative host pathophysiology between various malarias. This will be reserved until a later time when we have presented out studies in other primate malarias.

SUMMARY

1. Splenectomized and intact rhesus monkeys were infected with P. coatneyi. The course of infection was studied sequentially in terms of parasitaemia, haematology, blood chemistry, and tissue pathology.

2. Splenectomized Indian rhesus became acutely ill with the infection terminating in death in two of three animals. The acute infection was characterized by anaemia, haemoglobinuria, elevated transaminases, BUN and creatinine. The liver exhibited centrilobular necrosis and the kidney of one animal showed hyaline droplet degeneration.

3. Splenectomized Thai rhesus and intact Indian rhesus had milder infections. Anaemia was the outstanding abnormality. A vigorous response of the bone marrow was evidenced by reticulocytosis, normoblasts in the peripheral blood and erythroid hyperplasia. The haemogram returned towards normal with the onset of chronicity. Of the five moderately infected rhesus only one, a splenectomized Thai rhesus, had elevated transaminase, BUN and creatinine.

4. During the chronic phase no haematologic or blood chemistry abnormalities have been observed to date.

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TABLE I — Haematology and blood chemistry values of serial samples from control rhesus SP9

Day	Haematology					Blood Chemistry							
	WBC /mm^3	RBC x10^6 / mm^3	Retic–ulocytes %	Haemato–crit %	Haemo–globin gm%	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha-tase Sigma Units	BUN mg%	Creati–nine mg%	Choles–terol mg%
0	8,700	4.81	0.5	37	11.0	0	0.1	42	33	1.1	21.0	1.1	136
7	7,600	4.46	0.4	34	10.0	0	0.1	31	20	3.2	15.0	0.9	139
14	5,800	4.35	0.0	30	10.0	0	0.1	41	15	2.0	19.7	0.8	139
20	8,000	4.44	0.4	30	9.7	0	0	30	34	4.2	13.9	0.9	146
27	7,200	3.42	0.7	30	9.4	0	0.1	29	23	3.2	11.8	1.1	146
34	8,500	4.02	0.4	33	10.2	0	0.2	35	23	2.1	14.9	0.9	141
41	7,600	4.48	0.6	34	9.8	0	0	29	18	2.8	15.9	0.9	146
55	11,800	3.67	0.8	32	9.3	0	0	27	17	5.6	12.1	1.0	113
61	9,800	4.33	0.5	33	9.9	0	0.1	26	13	4.0	16.2	0.9	127
68	8,100	4.78	0.6	34	10.1	0.1	0.3	28	16	3.2	10.7	0.8	155
75	9,100	4.77	0.8	35	10.1	0.05	0.1	18	17	4.2	9.3	1.0	145
Range	5,800–11,800	3.42–4.81	0.0–0.8	30–37	9.3–11.0	0.0–0.1	0.0–0.3	18–42	13–34	1.1–5.6	9.3–21.0	0.8–1.1	113–155

TABLE II — Haematology and blood chemistry values of serial samples from control rhesus MS37

Day	Haematology					Blood Chemistry							
	WBC /mm³	RBC x10⁶ / mm³	Reticu-locytes %	Haemato-crit %	Haemo-globin gm%	Bilirubin mg%		SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha-tase Sigma Units	BUN mg%	Creati-nine mg%	Choles-terol mg%
						Direct	Total						
0	10,700	3.90	0.2	32	7.6		0	20	15	9.2	14.2	0.8	122
7	8,500	4.30	0.2	30	7.8		0.1	24	17	6.0	10.1		121
21	6,600	3.80	0.3	25	6.6		0.2	48			11.8	0.6	129
29	6,900	4.52	0.2	27	7.4	0.1	0.2	35	25		15.3	0.8	117
35	5,500	3.01	0.4	28	7.2	0	0.1	37	24	6.0	10.3	1.1	133
42	10,200	4.66	0.2	39	8.7	0	0	41	20		8.5	1.0	121
49	7,200	3.91	0.4	30	7.6	0.2	0.3	35	20		17.6	1.1	125
56			0.3	28	7.2	0	0.1	47	21	5.8	9.3	1.3	116
62	5,800	3.41	0.5	24	6.0	0	0.1	40	14	6.2	11.0	0.8	138
69	5,800	3.35	0	26	6.0	0.1	0.1	51	12	6.1	14.9	1.3	162
75	6,200	4.58	0.4	29	7.2	0	0	47	17	9.4	11.7	1.2	136
83	6,600	4.22	0.3	30	7.6	0	0	31	19	7.8	12.8	0.9	114
89	9,000	4.22	0.2	30	7.4	0.05	0.3	42	23	8.8	14.3	1.1	131
96	9,100	3.99	0.2	27	6.5	0	0	53	25	10.0	18.3	1.2	112
103	5,700	3.90	0.4	26	6.5	0	0.1	48	29	9.8	15.6	0.9	138
116	6,000	5.17	0.2	28	7.8	0	0	29	15	11.8	19.4	0.8	128
123	11,100	4.36	0.3	30	7.8	0.05	0.1	42	20	9.6	14.6	1.2	130
130	6,700	4.38	0.4	30	7.1	0	0	23	17	13.8	12.0	1.2	126
Range	5,500–11,100	3.01–5.17	0.0–0.5	24–39	6.0–8.7	0.0–0.2	0.0–0.3	20–53	12–29	5.8–13.8	8.5–19.4	0.6–1.3	112–162

TABLE III — Haematology and blood chemistry values of serial samples from control rhesus KL2

Day	Haematology					Blood Chemistry							
	WBC /mm³	RBC x10⁶ / mm³	Reticu-locytes %	Haemato-crit %	Haemo-globin gm%	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha-tase Sigma Units	BUN mg%	Creati-nine mg%	Choles-terol mg%
0	9,400	4.41	0.3	34	10.3	.1	.3	36	24		18.4	.8	154
11	6,100	4.05	0.3	34	9.7	0	.1	47	24	16.6	21.6	.7	154
18	7,000	3.52	0.2	33	9.1	0	.1	48	22	16.2	19.7	1.1	170
25	5,200	4.14	0.3	33	9.3	0	0	43	22	19.0	18.8	0.9	158
31						0	.1	38	25	17.6	19.3	0.8	162
37	5,800	4.28	0.6	32	9.2	0	.1	56	24	18.6	13.2	1.1	162
44	8,700	4.51	1.2	35	9.7	.05	.3	52	28	19.8	13.2	0.7	170
51	8,700	4.10	.4	34	9.5	.05	.1	41	23	15.2	14.4	0.9	170
58	8,400	4.65	1.3	28	9.4	.10	.2	47	24	11.7	17.9	0.8	162
65		4.40	.5	35	10.4	0	.1	31	17	18.2	17.9	0.9	144
Range	5,200–9,400	3.52–4.65	0.2–1.3	28–35	9.1–10.4	0–.1	0–.3	31–56	17–28	11.7–19.8	13.2–21.6	0.7–1.1	144–170

TABLE IV – Haematology and blood chemistry values of serial samples from control rhesus SP1

Day	Haematology					Blood Chemistry								
	WBC /mm³	RBC x10⁶ / mm³	Reticulocytes %	Haematocrit %	Haemoglobin gm%	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Units	SGPT S.F. Units	Alkaline Phosphatase Sigma Units	BUN mg%	Creatinine mg%	Cholesterol mg%
0	5,400	4.10	0.7	32	9.4	0	0	55	16	8.8	17.8	1.2	146
8	8,200	4.24	1.0	31	9.7	.1	.2	71	14	8.6	15.8	.9	138
15	8,100	4.70	1.3	32	9.4	0	.1	67	17	7.8	21.8	.7	133
21	6,000	4.37	1.4	34	9.9	0	.1	66	15		17.9	1.2	154
27	8,000	4.92	1.5	35	10.6	.05	.1	70	20	12.0	19.2	1.0	154
34	4,300	3.87	1.0	31	9.3	0	0	67	18	9.0	17.8	0.8	146
41	5,700	3.94	1.4	31	9.0	.2	.4	74	22	11.6	17.9	1.2	146
48	8,400	4.53	0.7	35	9.8	0	.1	66	24	8.2	19.4	.9	138
55	8,000	4.65	0.3	32	9.7	0	0	53	12	11.8	14.8	1.3	142
Range	4,300–8,400	3.87–4.92	0.3–1.5	31–35	9.0–10.6	0 - .2	0–.4	53–74	12–24	7.8–12.0	14.8–21.8	.7–1.3	133–154

TABLE V Haematology and blood chemistry values for monkeys prior to infection

Day	Haematology					Blood Chemistry							
	WBC /mm³	RBC x10⁶ / mm³	Retic-ulocytes %	Haemato crit %	Haemo-globin gm%	Bilirubin mg% Direct	Bilirubin mg% Total	SGOT S.F. Units	SGPT S.F. Units	Alkaline Phospha-tase Sigma Units	BUN mg%	Creati-nine mg%	Choles-terol mg%
KL14	10,900	4.0	1.4	32	9.3	.05	0.1	37	22	19.8	24.5	0.9	168
KL3	11,200	5.9	1.2	37	11.0	0	0.1	34	14	17.6	13.4	0.9	182
KL2	8,400	4.4	.5	35	10.4	0	0.1	31	17	18.2	17.9	0.9	144
KL12	15,800 12,500 11,400	5.1 5.7 4.4	.4 .3 1.7	36 37 36	10.1 10.5 10.1	.05 .05 0	0.2 0.1 0.1	43 52 35	26 23 19	17.0 17.6 17.6	19.2 16.7 15.0	1.1 1.0 1.2	178 178 184
KL13	9,200 6,600 5,700	4.8 5.2 5.4	.3 .5 .6	38 38 34	11 11.2 10.1	.05 0.1 —	0.2 0.1 0.1	48 73 60	28 26 25	21.6 24.0 22.0	14.5 17.4 14.7	1.3 1.3 1.0	162 172 160
KL1	13,900	5.1	1.0	38	10.4	—	0.1	46	31	15.8	15.1	0.9	162
SP2	20,600	5.0	—	35	11.3	—	—	—	—	—	17.0	0.6	—
SP4	11,200	6.3	—	—	—	—	—	—	—	—	15.9	—	—
SP5	7,000 8,400 7,000	5.1 4.7 5·6	— — —	33 36 36	10.2 10.2 10.2	— — —	— — —	— — —	— — —	— — —	15.2 13.2 13.3	0.9 0.9 —	— — —
SP1	8,000	4.7	0.3	32	9.7	0	0	53	12	11.8	14.8	1.3	142
SP9	8,700	4.8	0.5	37	11.0	0	0.1	42	33	1.8	21.0	1.1	130
MS18	6,400	4.5	1.0	33	8.9	0.1	0.2	40	29	6.6	16.0	1.2	105
MS37	10,700	3.9	.2	32	7.6	0	0	20	15	9.2	14.2	0.8	122
MS2	8,300	5.1	.5	40	12.0	0.2	0.4	48	33	6.4	13.4	1.8	162
Range	5,700– 15,800	3.9– 6.3	0.2– 1.7	32–40	7.6– 12.0	0– .2	0– 0.4	31– 73	12– 33	1.8– 24.0	13.2– 24.5	0.6– 1.8	105– 184

Fig. 1 Parasitaemia, haematologic and blood chemistries of KL13

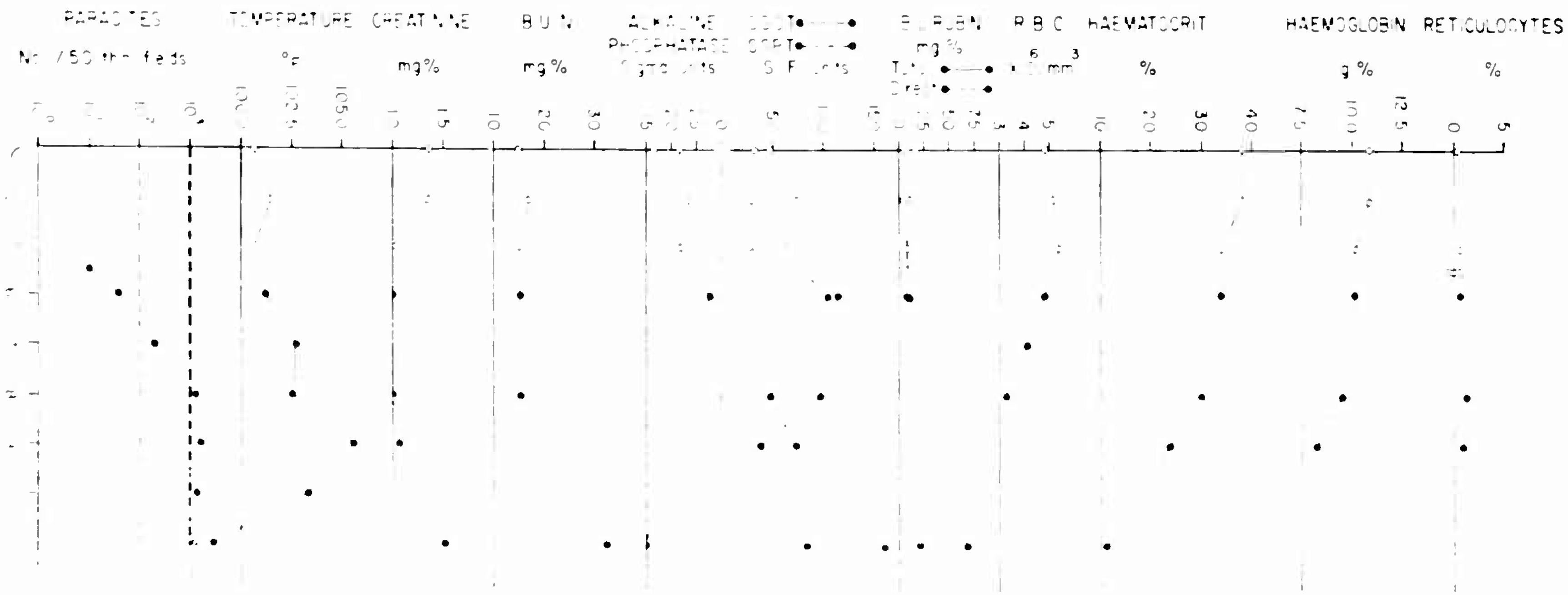
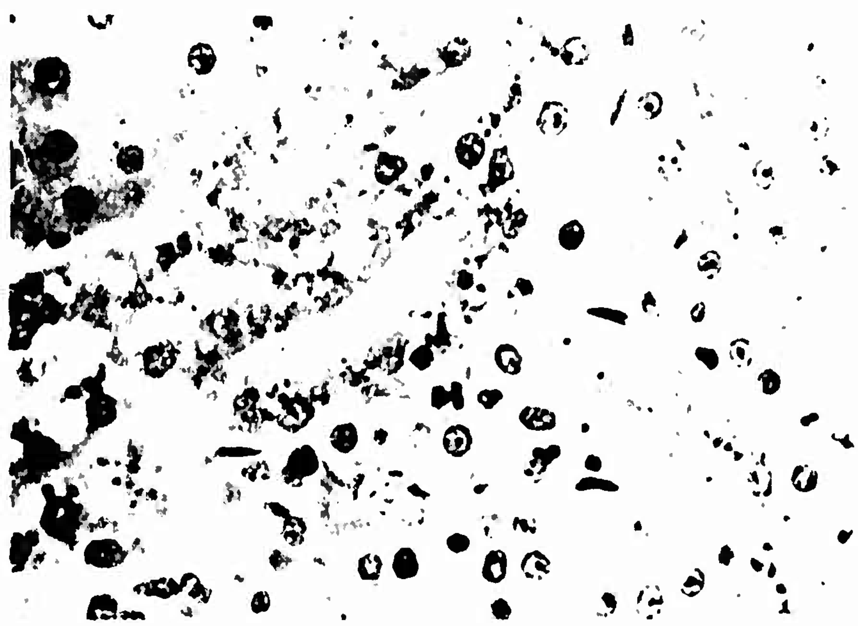


Fig. 2. Liver KL13 showing of centrilobular necrosis

Fig. 3. Kidney of KL13 showing hyaline droplet degeneration, hemoglobin casts and fluid in Bowman's space



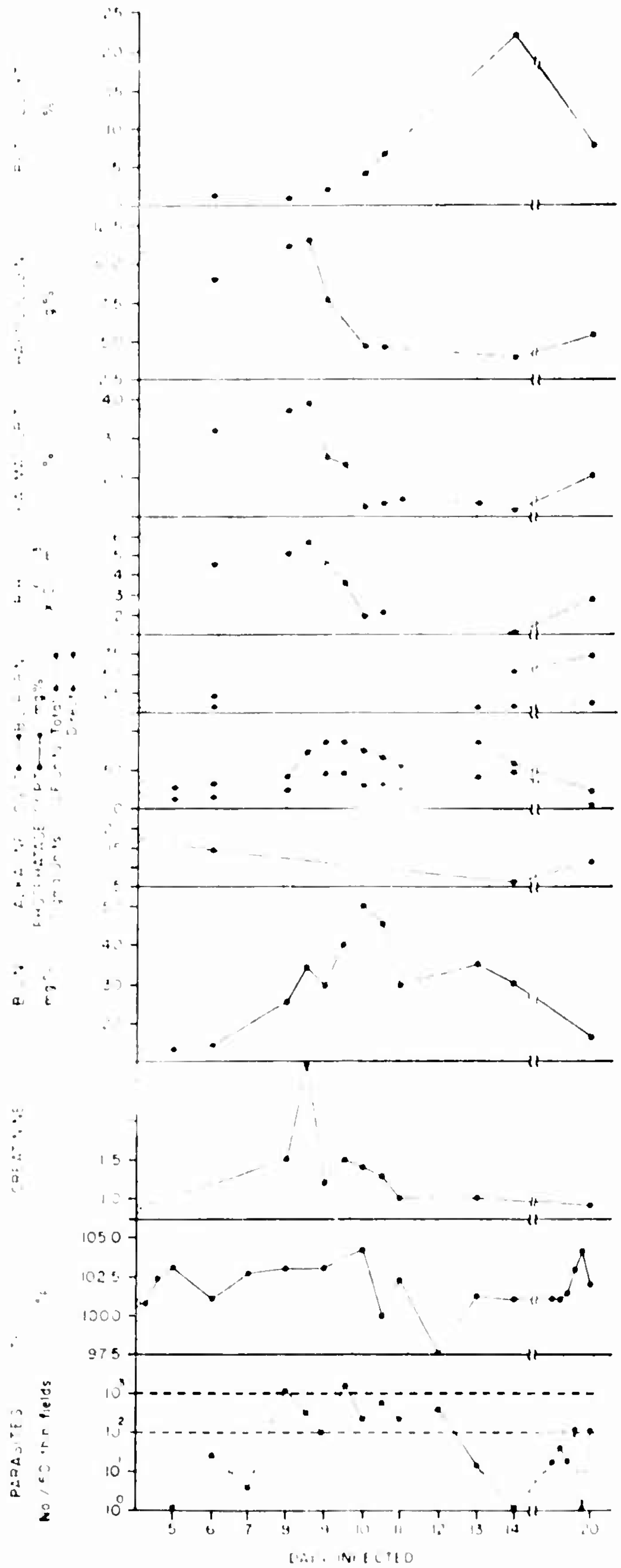


Fig. 4. Parasitaemia, haematologic and blood chemistries of KL3

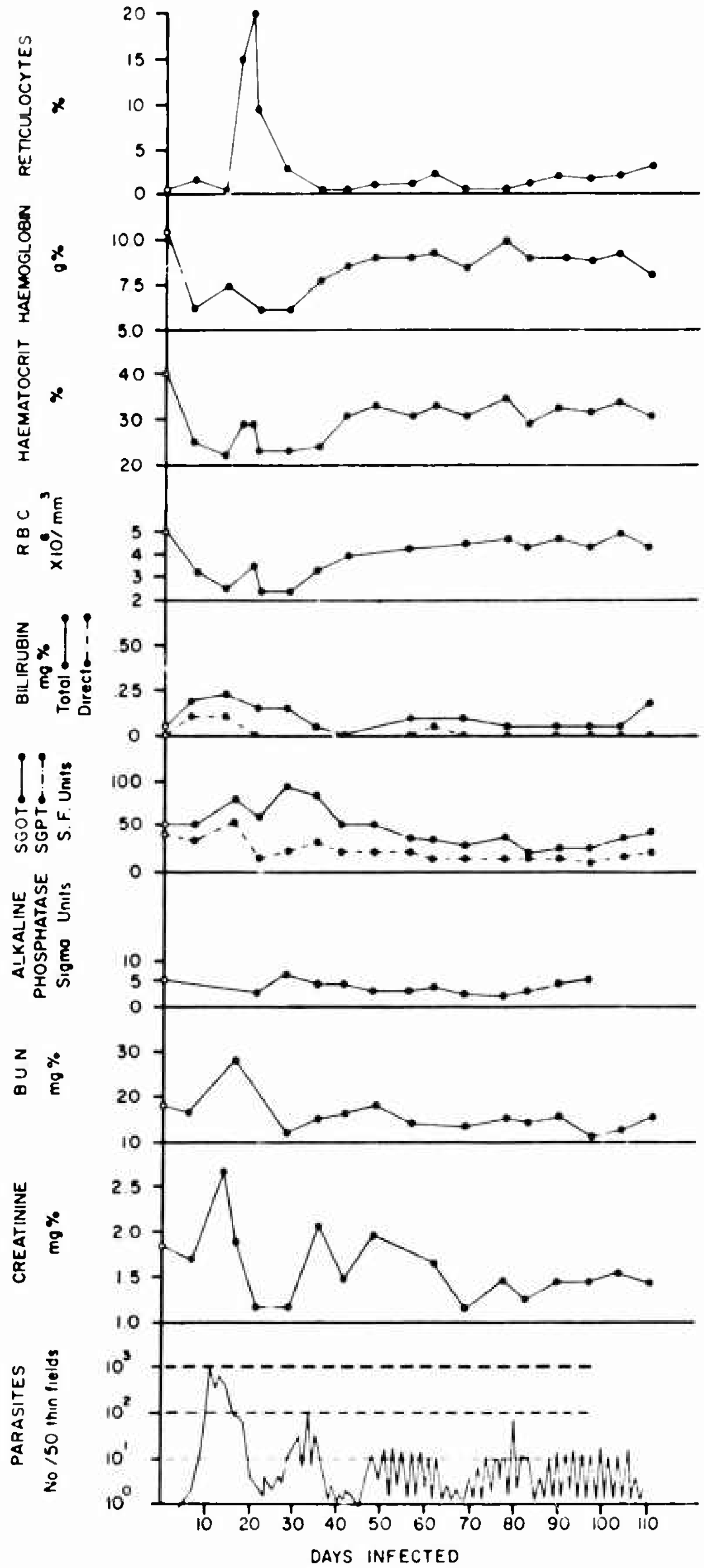


Fig. 5. Parasitaemia, haematologic and blood chemistries of MS2

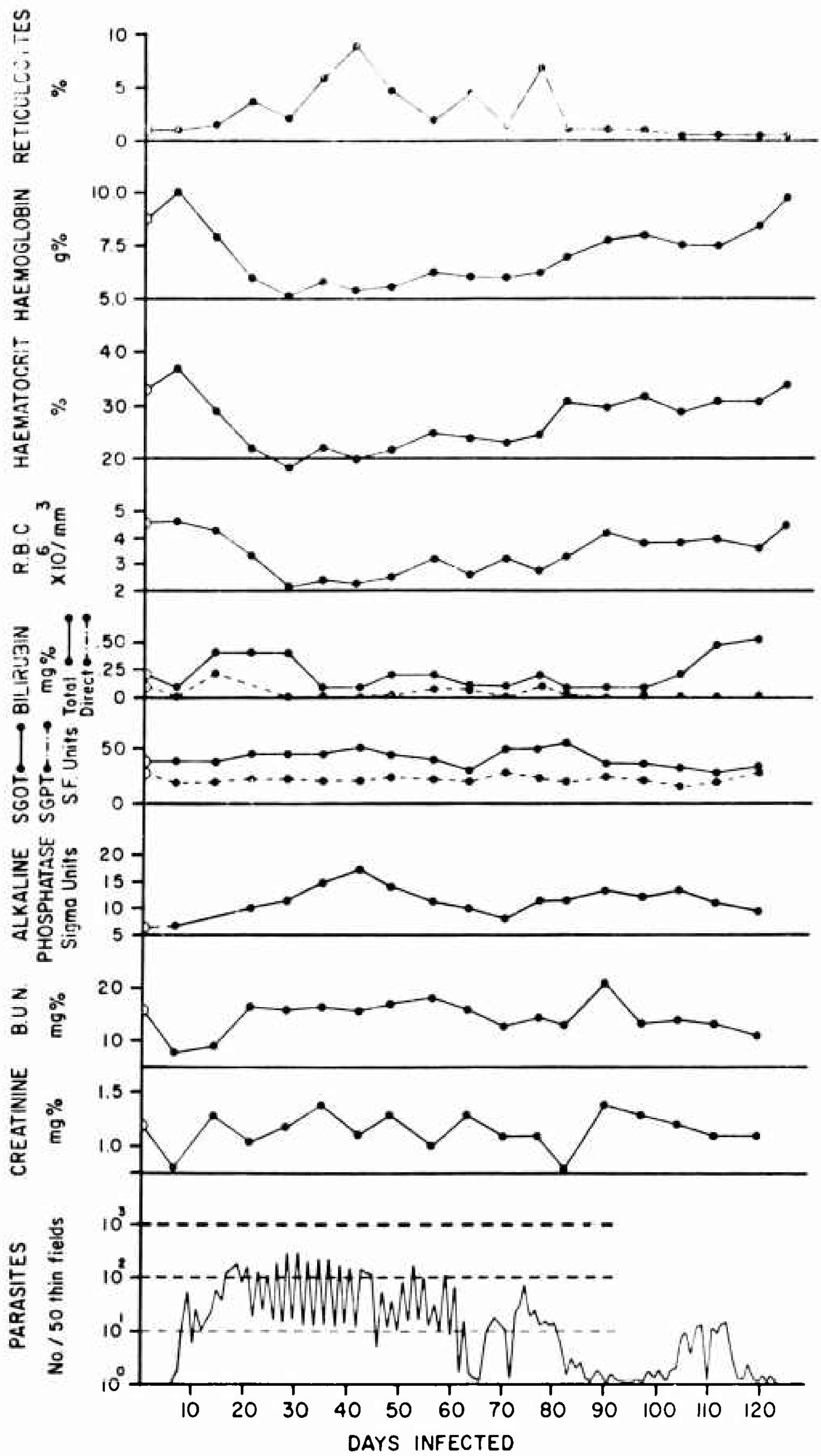


Fig. 6. Parasitaemia, haematologic and blood chemistries of MS18

Fig. 7. Parasitaemia, haematologic and blood chemistries of KL1

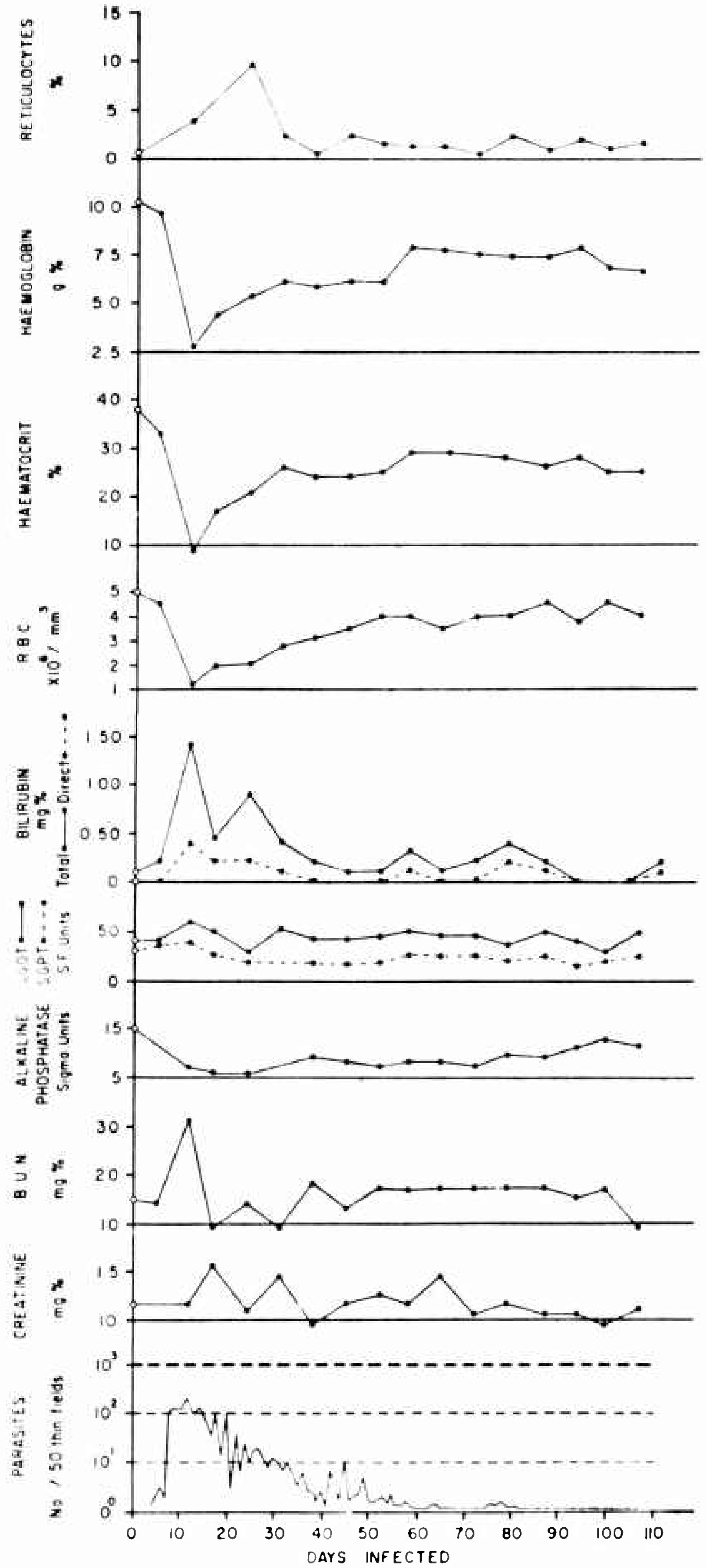
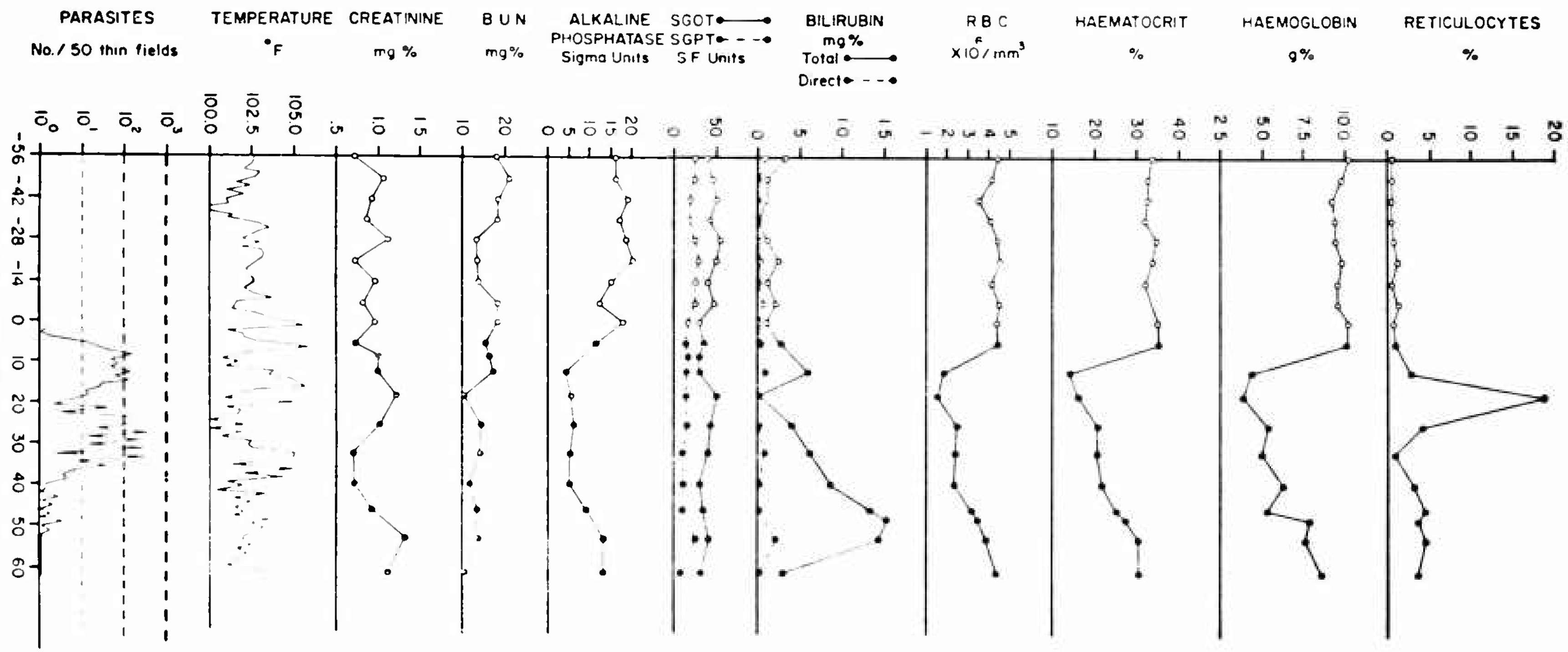


Fig. 8 Parasitaemia, haematologic and blood chemistries of KL2



Subtitle: Comparative Studies in the Pathology and Host Physiology of Malarias. Serum Protein Alterations in P. Coatneyi Malaria. A comparison of Cellulose Acetate and Polyacrilamide Disc Electrophoretic Patterns.

Investigators. Robert S. Desowitz, Katchrinnee Pavanand, Duangduen Vacharaphorn

Numerous observations have been made on the alterations of serum proteins during the course of malaria. The subject was reviewed by Stauber (1954) and the more recent knowledge summarized by Sadun et al (1966). In general, it has been found that gamma globulin increases, albumin decreases, and there is no consistent pattern of change in the alpha and beta globulins.

This present paper describes changes in serum proteins during P. coatneyi malaria of rhesus monkeys. In a previous paper in this series (Desowitz et al, 1967) it was shown that this parasite produced a wide spectrum of disease ranging from acute fatal to mild chronic. It was of interest, therefore, to determine whether the different types of infection would produce characteristic derangements in serum protein components.

Two methods of serum protein analysis have been employed in this study; conventional electrophoresis on cellulose acetate and disc electrophoresis. The latter is a relatively new technique in which separation is dependent not only on electrical charge but also the weight of the molecule. It will be seen that alterations are evident by disc electrophoresis that are not detectable by conventional zone electrophoresis.

METHODS

The course of infection and concommittant serum chemistry, haematologic, and histologic alterations for all infected rhesus monkeys were described previously (Desowitz et al, 1967). An aliquot of serum was obtained from all bleedings, usually at weekly intervals, for the estimation of total protein by the method of Weichselbaum (1946) and for thymol turbidity by the technique of Shank and Hoagland (1946). Microzone electrophoresis on cellulose acetate strips was performed and analyzed by the Spinco Analytrol apparatus and polyacrilamide disc electrophoresis by the Canalco model 12 (Canalco Co., Rockville, Md) and then scanned with the Densicord Model E densitometer (Photovolt Corp., New York).

RESULTS

Zone electrophoresis:

Serum proteins, as measured by cellulose acetate zone electrophoresis, for four representative infections are shown in fig. 1. Also illustrated in this figure are the course of parasitaemia and serum thymol turbidity. In all animals studied there was an increase in gamma globulin. In the Indian rhesus (KL series) per cent increase over the preinfection value ranged between 140% to 200% while in the Thai rhesus (MS and SP series) the increment was from 40% to 70%. In both groups there was no appreciable rise in gamma globulin during the first 20 days of infection, the period of the primary parasitaemic attack. In the Indian rhesus it increased with relative rapidity after this period while in the Thai rhesus, as will be seen from fig. 1, the incremental slope was much more gradual. The increase in thymol turbidity seemed to reflect the development of the hypergammaglobulinaemia. The rise in gamma globulin and thymol turbidity were, approximately, concurrent and proportional. There also appeared to be a relationship between gamma-globulin level and the course of the parasitaemia. In the Indian rhesus, which exhibited a rapid and marked rise of gamma-globulin, the primary attack was terminated about 30th to 40th day and the parasitaemia was scanty thereafter. In contrast, as noted previously, gamma-globulin increase in Thai rhesus was more gradual and lower. In these animals the primary parasitaemia usually persisted somewhat longer and the parasites were more numerous during the chronic phase.

In all animals, the beta globulin increased to varying degrees. Usually this rise was concurrent with that of the gamma-globulin. During the first 30 to 40 days of infection the alpha-globulin also rose. In some animals this was of a transient nature while in others the elevation persisted throughout the entire period of observation. Albumin decreased in all animals during the primary parasitaemia. There was a gradual recovery toward normal or greater than preinfection levels in the Thai rhesus while in the Indian rhesus the albumin level tended to remain depressed during chronicity.

Disc electrophoresis

Unlike conventional zone electrophoresis, disc electrophoresis is dependent upon the combined effect of molecular sieving in a polyacrilamide gel matrix and electrophoresis in a discontinuous buffer system. This resolves many more protein fractions than do the zone techniques. Eventually a new taxonomy of serum proteins may have to be devised to accomodate this new method. Several workers have attempted to correlate disc and electrophoretic fractions but there is still considerable confusion. Fig. 2 attempts to summarize the identification of disc serum electrophoretic components as proposed by a number of investigators. The regions of 19S and 7S globulins have been localized with reasonable certainty although both components show considerable heterogenity. This is particularly true of the 7S globulin region. The identification of the IgM is still tentative and is based upon disc electrophoresis of a fraction isolated by column chromatography (Desowitz and Russell, unpublished results). The transferrin (β_1-globulin region) was, in some animals composed of two distinct components.

Disc electrophoresis of serial serum samples from infected monkeys evidenced certain progressive alterations not detectable by the conventional zone technique. The most notable of these changes was the consistent increase in macroglobulins in all monkeys studied. The major macroglobulin elevation was in the fraction tentatively identified as IgM. The increase in IgM occurred approximately between the 25th and 40th day although one animal, MS2, the elevation was not detected until the 77th day. The gradual increase of IgG, occurred concurrently with that of the IgM. The transferrin component also increased and this probably reflected the rise in β-globulin noted in the zone electrophoretograms. Alterations in post albumins (probably α 1 components) varied from animal to animal as it did in the zone technique. Two typical examples of disc electrophoretograms of serial serum samples are shown in fig. 3.

DISCUSSION

The increase in gamma-globulin during P. coatneyi conforms to the general picture of hypergammaglobulinaemia described for many other malaria infections. Disc electrophoresis revealed increased concentrations of IgM and IgG although as would be expected the major moitey of the immunoglobulins was of the latter type. There seemed to be a discernible causal relationship between gamma globulin production and the course of parasitaemia. Kuvin et al (1962), Abele et al (1965), Lunn et al (1966) and Tobie et al (1966) have made similar observations on the influence of immunoglobulin level on the parasitaemia. Although the notion may be heretical in this era of sophisticated serology, we suggest that the direct measurement of the immunoglobulins may be as good as or a better indicator of functional immunity than many of the available serologic techniques. A number of studies such as that by Lunn et al (1966) have revealed a discrepancy between gamma globulin and antibody levels and that the immunoglobulin concentration seemed to represent a more rational causal relationship to the immunologic factors affecting the dynamic balance of the host-parasite system. An additional deficiency in present day serology applied to malaria is that the techniques in common use do not seem to measure, directly, protective antibody (Targett and Voller, 1965; Mahoney et al, 1966).

While the role of the 7S immunoglobulins in malaria has been well defined by McGregor and his colleagues (Cohen et al, 1961; Cohen and McGregor, 1963; McGregor et al, 1963) less is known of the macroglobulins participation in the immune response. It has recently been demonstrated that there was a marked increase of IgM during the primary attack and relapses of volunteers infected with P. vivax and P. cynomolgi (Tobie, 1965; Abele et al, 1965; Tobie et al, 1966). Elevated IgM has also been found in the sera of people living under conditions of hyperendemic malaria (Turner and Voller, 1966). This present study

employing disc electrophoresis has also shown this increase in immunomacroglobulin. We have also found, in confirmation to Tobie et al: (1966) observation, that during the primary attack both IgM and IgG show a coincidental elevation. As Tobie et al have pointed out, this finding is contradictory to the accepted concept of sequential immunoglobulin synthesis. The functional role of IgM in malaria is still unknown. While it does not seem to possess protective properties (Cohen et al, 1961) the sephadex gel filtration IgM fraction was serologically positive with the fluorescent antibody test. It should be pointed out, however, that we have found by disc electrophoresis, contamination of IgG in similarly isolated fractions of IgM and this may be a least partly responsible for the serologic reaction. There is also the interesting suggestion by Houba and Alison (1966) that some of the IgM in people living in hyperendemic areas may be of a type related to rheumatoid-factor-like macroglobulin. Obviously, better serologic techniques which are truly indicative of the various functional immune mechanisms will have to be developed before the roles of the different immunoglobulins can be determined.

Despite the often repeated observation of lowered serum albumin during the clinically active stages of malarias little is known as to the underlying causative mechanism. Maegraith (1948) has hypothesized that the fall in albumin concentration is due to a functional hepatic derangement in albumin synthesis. However, in the absence of any data on total albumin pool, rates of synthesis and turnover it is not possible to explain the hypoalbuminaemia. It should be noted that in our animals there was no significant loss of albumin in the urine (Desowitz et al, 1967).

SUMMARY

Alterations in serum proteins during the course of P. coatneyi malaria in rhesus monkeys have been measured by the cellulose acetate zone and polyacrilamide disc electrophoretic methods. Zone electrophoresis evidenced elevations of gamma, beta and alpha globulins during the course of infection. Albumin decreased during the primary infection. Disc electrophoresis indicated a concomittant increase of IgG and IgM after the 25th day of infection.

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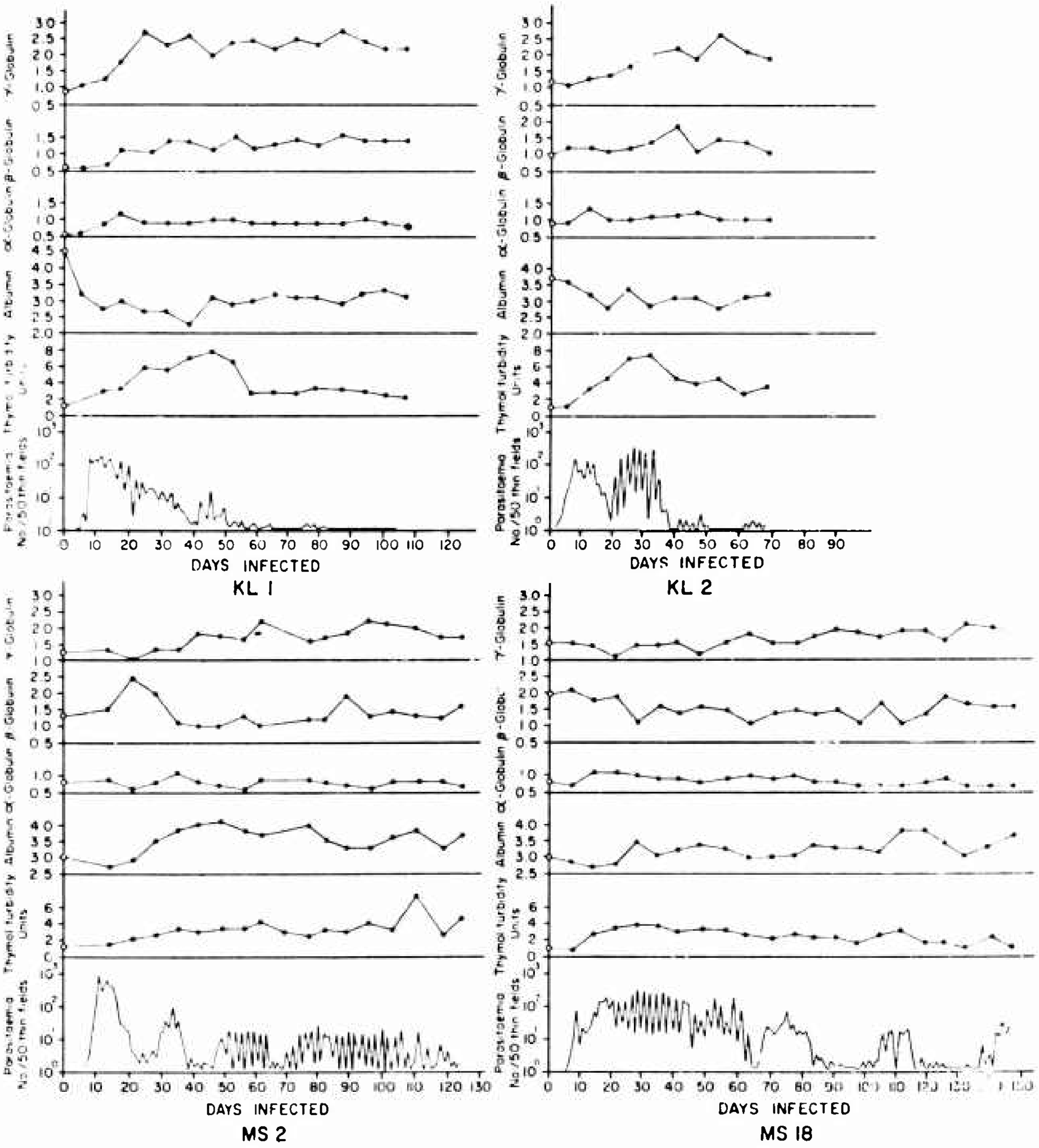
Fig. 1: Serum proteins and parasitaemia during the course of four representative *P. coatneyi* infections



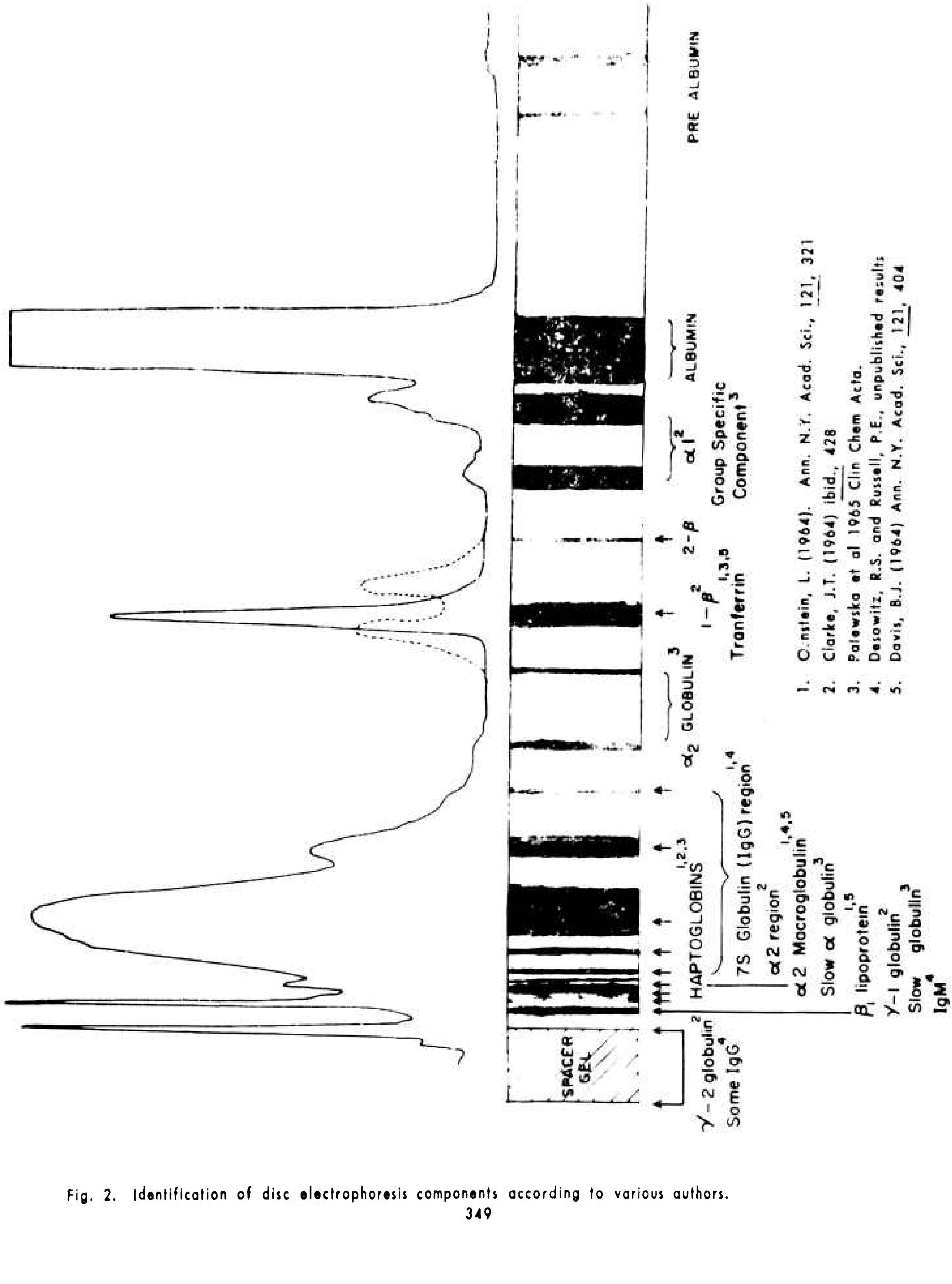
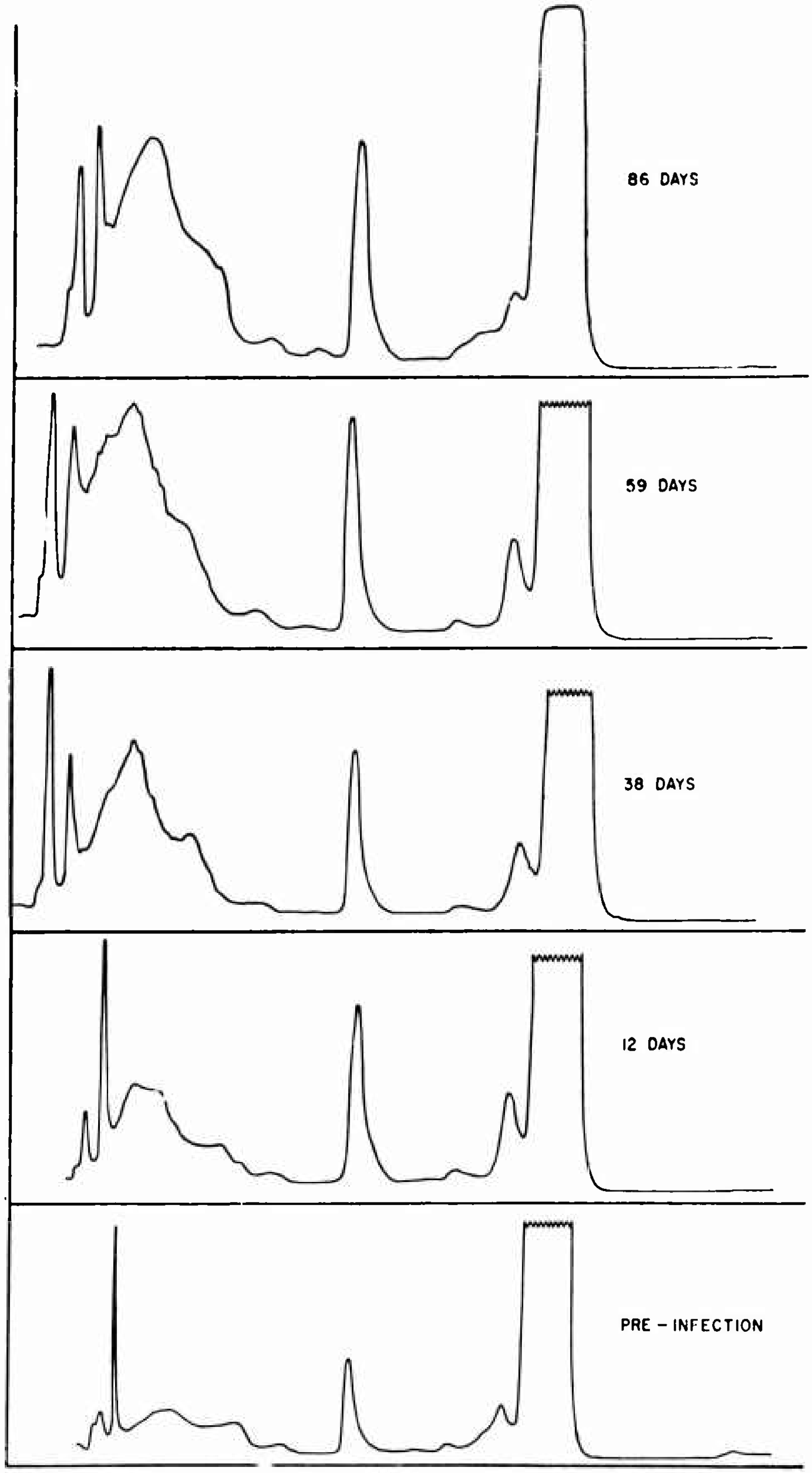
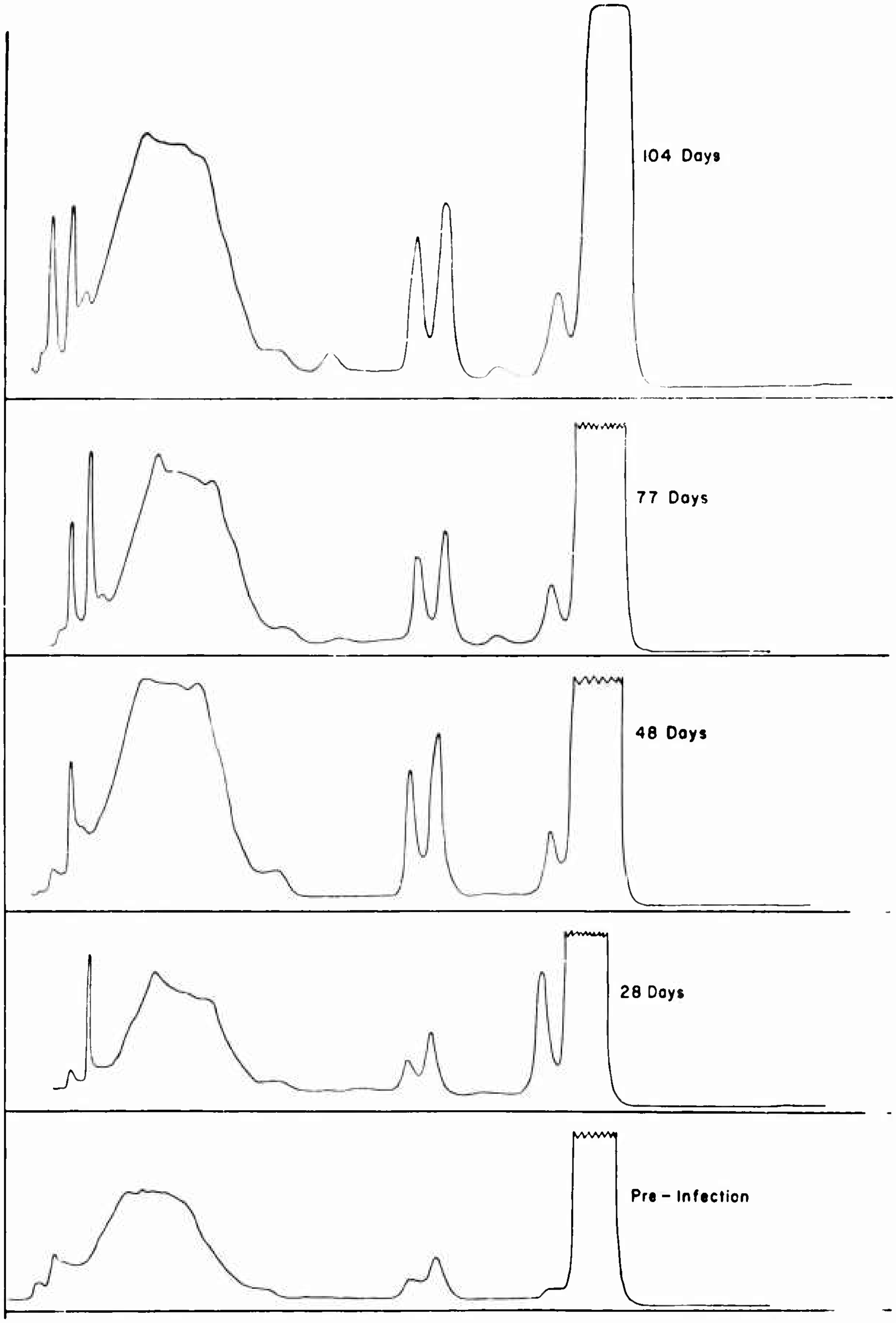


Fig. 2. Identification of disc electrophoresis components according to various authors.

Fig. 3. Disc electrophoretic changes during the course of *P. coatneyi* infections




104 Days
77 Days
48 Days
28 Days
Pre - Infection

Subtitle:

COMPARATIVE STUDIES IN THE PATHOLOGY AND HOST PHYSIOLOGY OF MALARIAS. PLASMODIUM INUI MALARIA.

Investigators: Robert S. Desowitz, Louis H. Miller, Richard D. Buchanan, Vithune Yuthasastrkosol, and Barnyen Permpanich.

Preceding reports of this series described the pathophysiology of Plasmodium coatneyi and gibbon malarias. This report is concerned with the infection caused by P. inui in Macaca mulata. P. inui is of interest as potential model for human quartan malaria which it resembles in morphology and schizogonic behavior. Another point of affinity is that P. inui is capable, at least under experimental conditions, of infecting man (Coatney et al, 1966).

While the data are complete for the P. inui study they have not, as yet, been fully analyzed. In view of this only a summarization of our findings is given in this report.

Methods

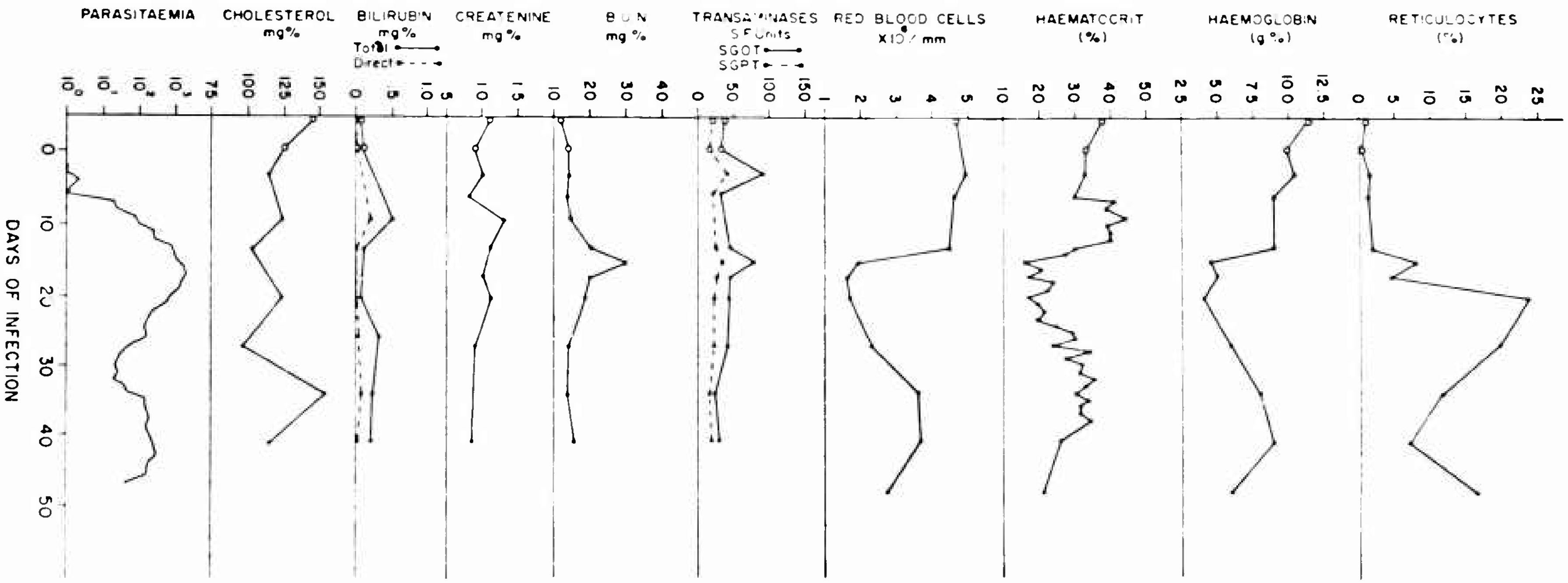
The strain of parasite used in this study was isolated from a naturally infected M. irus that came from S. Thailand (see Ann. Rep., 1966). It is probably P. inui var. shortii. The methods of study followed that for P. coatneyi (Desowitz et al, 1967).

Results

As in P. coatneyi malaria there was a wide variation in intensity of infection. The most severe infections were produced in splenectomized rhesus monkeys although only in one instance did the animal die. A typical course of infection across the acute phase (first 40 days) is shown in fig. 1. It will be seen that prior to the haemolytic phase there is, early in the infection, a haemoconcentration phase. This was also described for P. coatneyi infections but preliminary results indicate that the period of haemoconcentration may be somewhat more prolonged in P. inui malaria. During the primary parasitaemia in splenectomized rhesus there was evidence of hepatic and renal pathophysiology. Transaminases (SGOT), BUN and creatinine were all elevated. These elevations were usually of a transient nature and the serum chemistries returned to normal by the 25th day despite a continuing moderate parasitaemia. Cholesterol also was lowered during the acute phase when a pronounced anemia was evident. However the hypocholesterolaemia was not as marked as that in gibbon malaria (Miller et al 1967).

A number of infections have now been followed for one year or more which has permitted a study on the effect of chronic P. inui. Unlike P. coatneyi in which only a very scanty parasitaemia was found after about the 60th day, P. inui often persisted in moderate numbers throughout the entire observation period of a year or more. Two animals sustained a parasitic recrudescence between the 150th and 200th days. As a consequence of this attack there was marked evidence of renalhepatic pathology. The renal pathology was of special interest since it showed some similarity to nephrotic syndrome i.e., elevated BUN, creatinine and cholestorol and concomittant proteinuria (fig. 2). It has been postulated that chronic P. malariae infections may cause nephrotic syndrome. There has been no direct evidence for this and the hypothesis has been based on epidemiologic observations. If further experiments confirm that chronic P. inui may also cause a similar pathology then a highly useful model would be available.

Fig. 1. Course of parasitaemia, haematology and serum chemistries during the course of a P. inui infection in a splenectomized rhesus



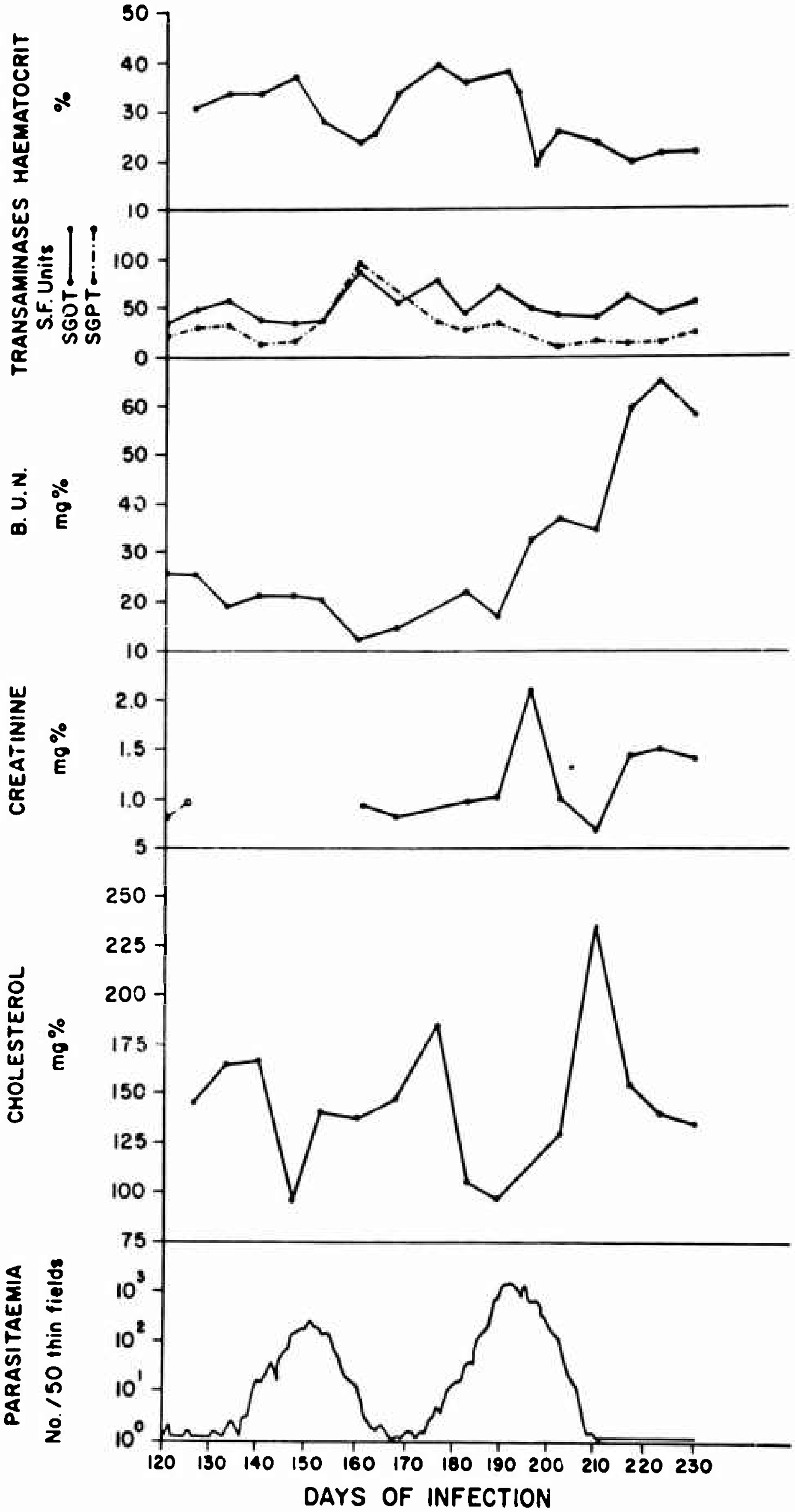


Fig. 2. Haematology and serum chemistries during a late parasitaemic recrudescence in a *P. inui* infected rhesus.

Subtitle: A Vascular Permeability Increasing Factor in the Serum of Monkeys Infected with Primate Malarias

Investigators: (R.S. Desowitz and Katchrinnee Pavanand)

A number of biological substances, chiefly certain globulins and polypeptides, have been shown to cause increased vascular permeability. However, while there has been a considerable amount of work in identifying and characterizing the nature of these substances, little is known regarding the possible role they may play in disease.

Goodwin and Richards (1960) reported the presence of pharmacologically active peptides in the blood and urine of mice infected with Babesia rhodaini and Plasmodium berghei. These peptides which stimulated the guinea-pig ileum and rat duodenum increased in amount as the infections progressed. It has also been shown that the amount of bradykinin, one of a related group of polypeptides that can cause an increased vascular permeability, is abnormally high in rhesus monkeys infected with P. knowlesi (Tella and Maegraith, 1962, 1963).

Both Knislely, et al (1945) and Overman and Feldman (1947) claimed that there was an alteration of body fluid compartment physiology in fatal P. knowlesi infections. Since there appears to be an increase of pharmacologically active substances in the blood of malaria infected animals it is possible that they might be partly responsible for this change in body fluid compartmentalization. This present paper describes a vascular permeability increasing factor (PIF) in the sera of malaria infected rhesus monkeys.

Methods

The sera of rhesus monkeys infected with P. inui or P. coatneyi were employed in these experiments. The principles of laboratory animal care as promulgated the National Society for Medical Research were observed.

Results

The results of these experiments are shown in figs 1-5. Normal, undiluted serum from all monkeys produced a vascular permeability effect. With the exception of SP2 whose serum was active at 1:10, PIF activity of the pre-infection serum disappeared at the 1:10 dilution. In all monkeys the PIF activity of the diluted serum became apparrent with the onset of patency. There appears to be some relationship between the degree of PIF activity and the nature of the parasitaemia. MS23 and SP2, (figs 1 & 2) sustained a similar course of P. inui infection. In both animals the primary peak parasitaemia did not exceed 5 per cent. This was followed by a period of chronicity during which time several recrudescences occurred. PIF activity increased during the period of primary parasitaemia and then disappeared about the 80th day of infection. During or following a recrudescence there was a reappearance of the PIF in the serum.

The pattern of PIF activity was somewhat different in the two P. inui infected monkeys, SP4 and MS24, that sustained an acute primary parasitaemia in which over 20 per cent of the erythrocytes were infected (figs 3 & 4). In both these animals PIF activity was reduced before the point of peak parasitaemia. The infection terminated fatally in MS24 but in SP4 the animal survived and the infection became chronic; again, serum PIF activity reappeared coincidentally with parasitaemic recrudescences.

The single P. coatneyi infected monkey also showed a serum PIF (Fig. 5) but unlike the P. inui infections the level of activity did not decrease during the chronic phase of the infection.

It was found that 10 mg/kg phenergan given to rabbits prior to intredermal inoculation of serum completely blocked PIF activity.

Discussion

It is has been shown that the serum of monkeys infected with P. inui and P. coatneyi cause an increased vascular permeability when inoculated intradermally into the skin of the white rabbit. The permeability increasing activity of the serum was completely blocked by 10 mg/kg of an antihistamine, phenergan.

The factor responsible for causing the increased vascular permeability has not as yet been identified but may be a pharmacologically active peptide identical or related to the substances reported by Goodwin and Richards (1960) and Tella and Maegraith (1962, 1963). Whether the PIF is the same substance that is present in normal uninfected serum in low concentration or is a new substance that appears only with infection also is unknown. The possibility exists that the PIF may be induced by antigen-antibody complexes. That this may occur in parasitic infections has already been suggested in the report of the WHO expert committee on immunology and parasitic diseases (1965). Recently Ward and Conran (1966) demonstrated the presence of malaria antigen, γ-globulin and BIC-globulin deposited along the endothelial surfaces of the renal glomerulus. These authors suggest that the deposite might be antigen-antibody complexes that would cause a local increase of vascular permeability.

Further evidence of increased vascular permeability in malaria has been given by Malloy (pers. comm.) who found an alteration in fluid compartment physiology of P. falciparum-infected American soldiers in Vietnam. Our investigations on host physiology in primate malarias, of which a detailed account will be presented in subsequent communications, has shown a liver pathology with centrilobular necrosis in some P. coatneyi infected rhesus. It is possible that a PIF could cause a rapid decrease in plasma volume which might, in turn, induce shock and the consequent pathologic change in the liver. This is still highly conjectural but it would seem important to elucidate the possible participation of a PIF in the pathogenesis of malaria.

Summary

1. The sera of monkeys infected with P. inui or P. coatneyi caused an increased vascular permeability when inoculated into the skin of rabbits.

2. PIF activity in P. inui infected sera was reduced during the chronic stage of the infection but reappears during recrudescences. In acute infections a decrease in activity was observed immediately before or during the height of the primary parasitaemia.

3. The activity of infected sera was blocked when an antihistamine, phenergan was given to the rabbits.

Fig. 1. The course of infection and serum vascular permeability increasing activity in a P. inui infected rhesus (MS23)

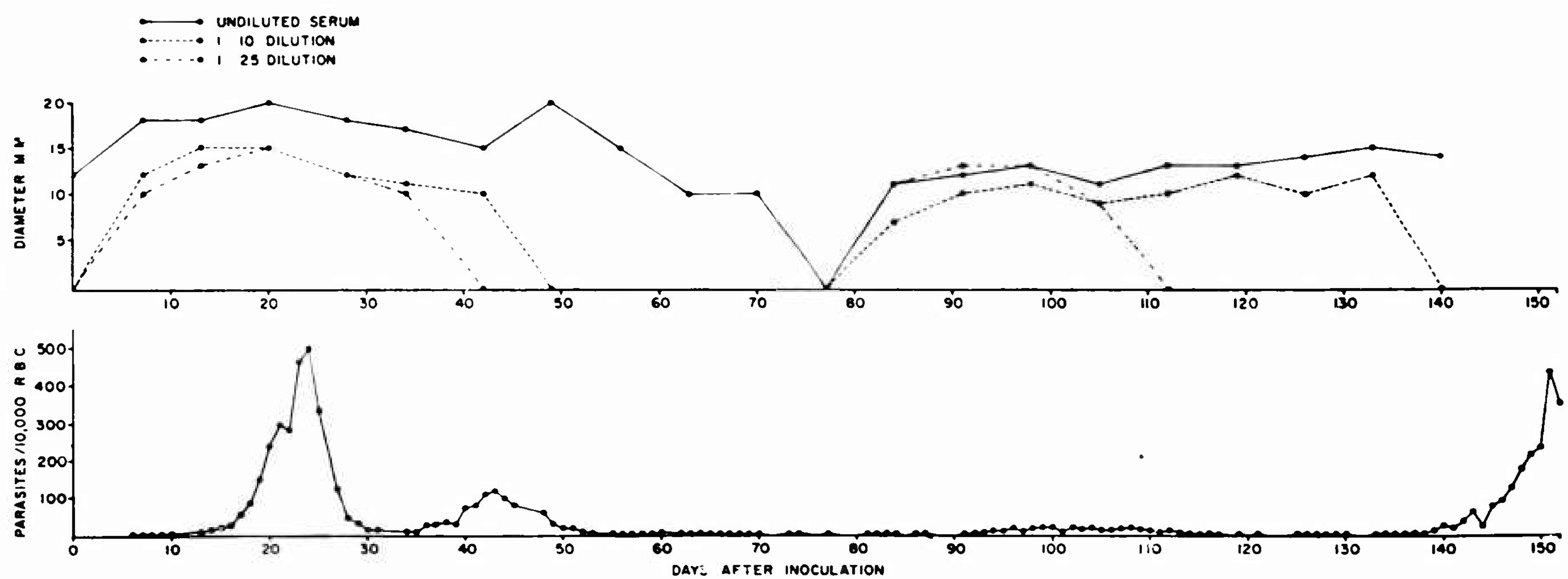


Fig. 2. The course of imfection and serum vascular permeability increasing activity in a P. inui infected rhesus (SP2)

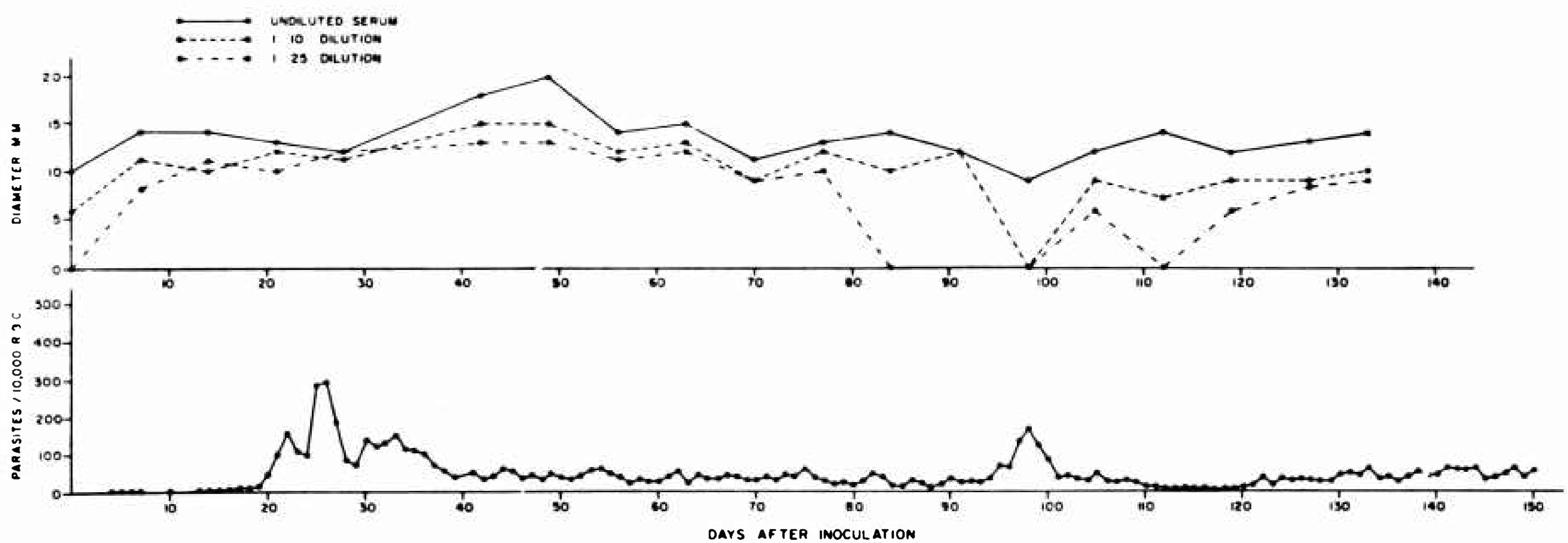
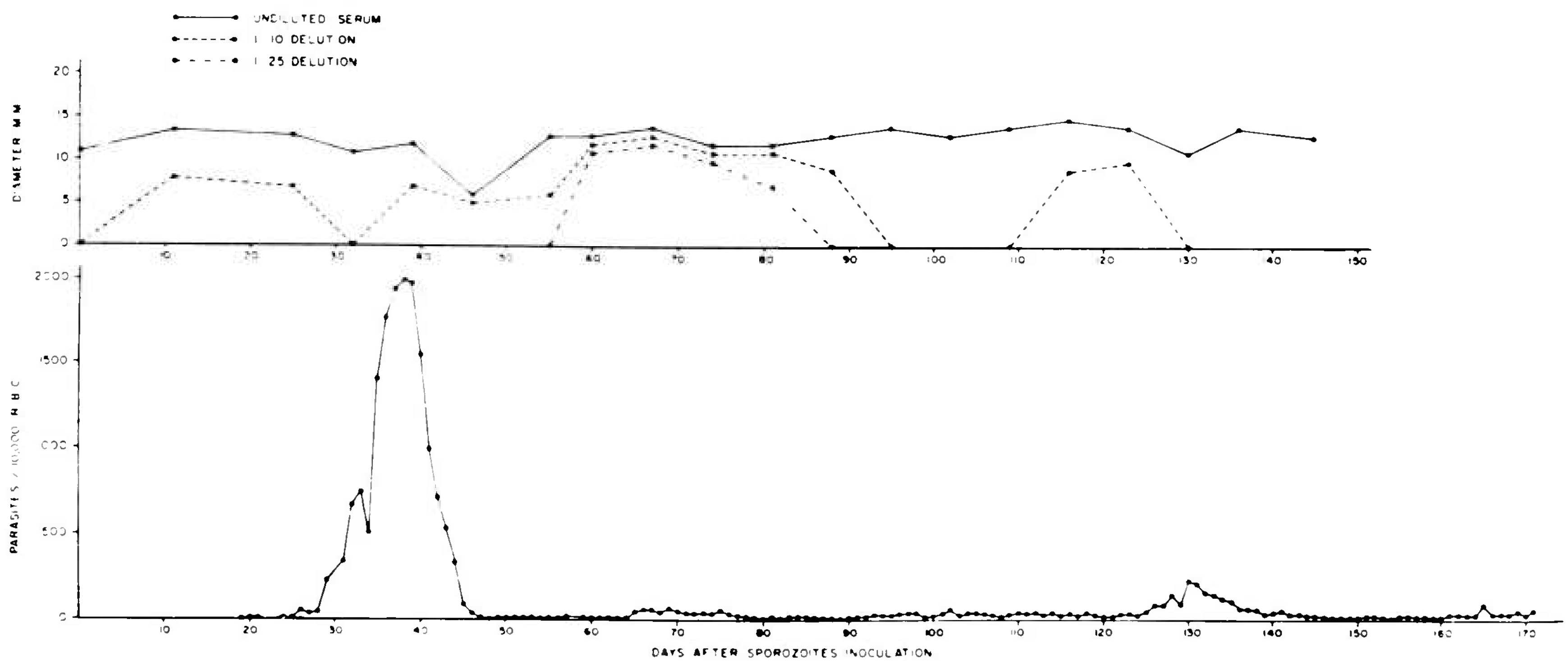


Fig. 3. The course of infection and serum vascular permeability increasing activity in a P. inui infected rhesus (SP4)



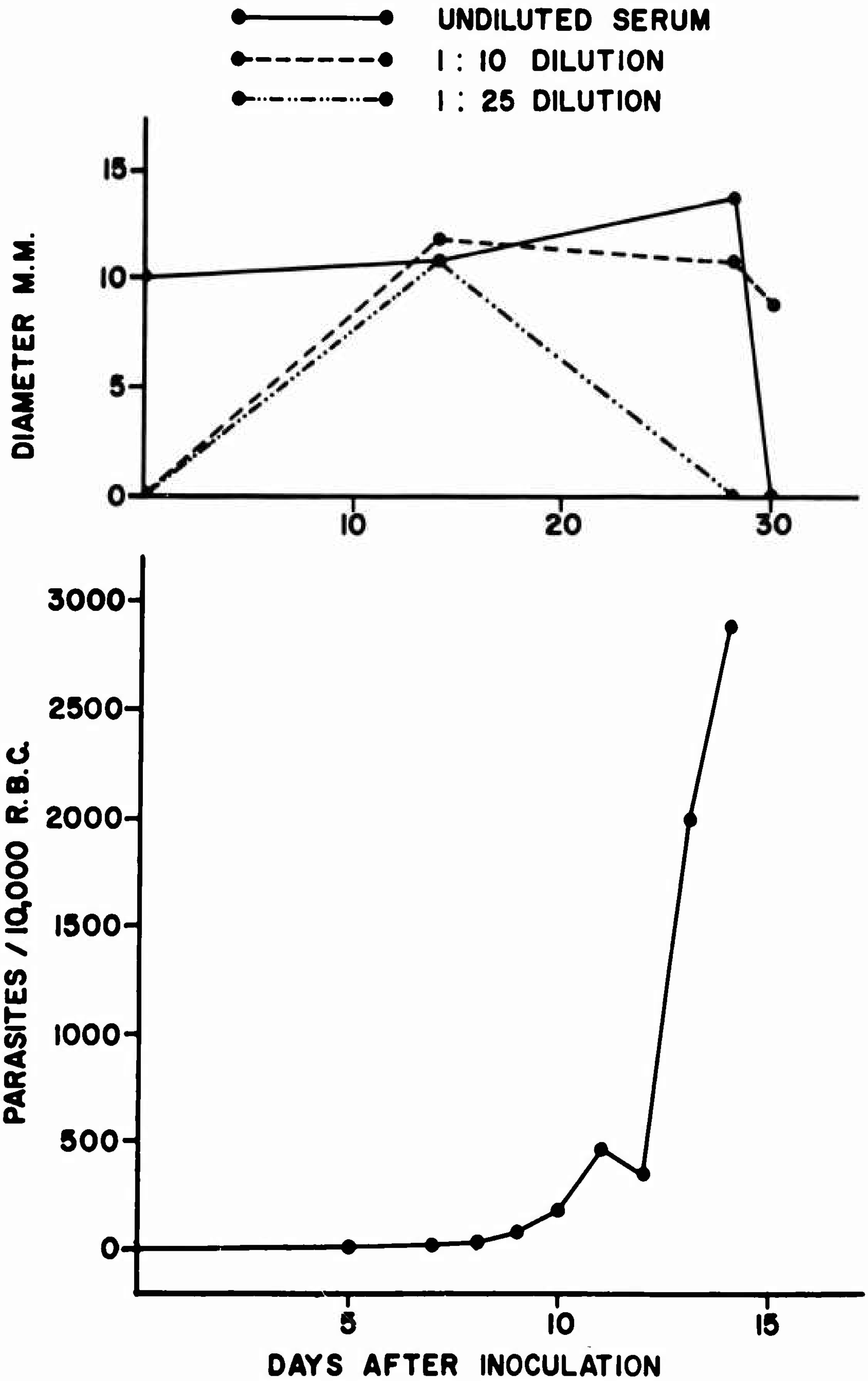
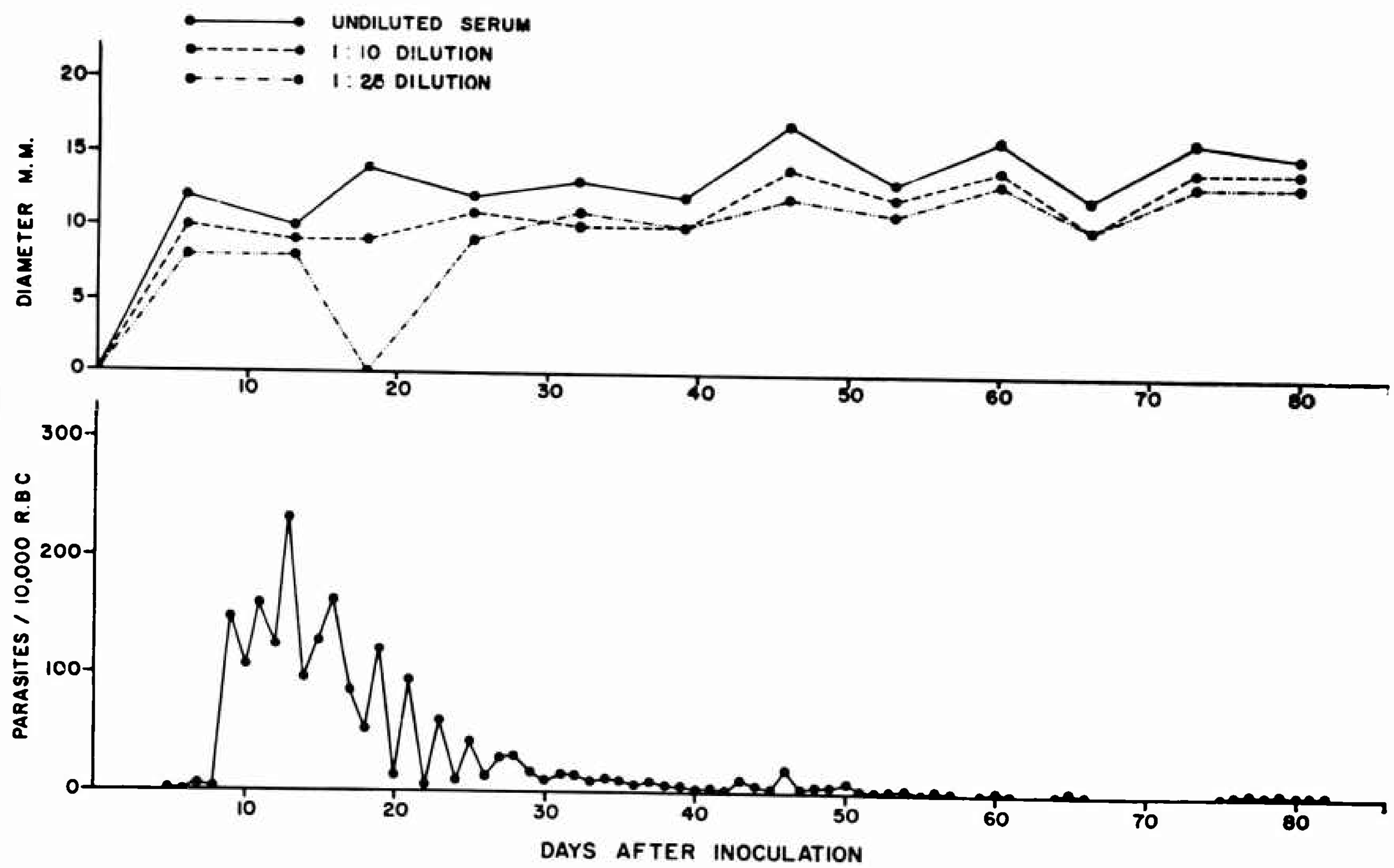


Fig. 4. The course of infection and serum vascular permeability increasing activity in a P. inui infected rhesus (MS24)

Fig. 5. The course of infection and serum vascular permeability increasing activity in a P. coatneyi infected rhesus (KL1)



Subtitle: Comparative Studies in the Pathology and Host Physiology of Malarias: Renal Function in Mice Infected with P. berghei

Investigators: Louis H. Miller, Katchrinnee Pavand, Richard D. Buchanan, Robert S. Desowitz, Eam Athikulwongse

Although acute renal failure is one of the important complications of falciparum malaria, the underlying mechanism (s) has not been fully elucidated. Early workers attributed acute renal failure of malaria to haemoglobin casts in the renal tubules. This explanation was refuted by Foy et al. (1943) and Maegraith and Findlay (1944) who failed to find obstructing casts in the kidneys of malaria patients dying from renal failure. Maegraith (1944) suggested that in common with other conditions such as crush injuries, incompatible transfusions, concealed accidental hemorrhages and cholera, tubular necrosis in malaria results from a shunting of blood from the cortex to the medulla. The recent work of Chongsuphajaisiddhi (1966) in P. knowlesi infected rhesus monkeys supports this concept. By renal artery angiography he demonstrated a marked reduction in perfusion of the kidney, more evident in the cortex than the medulla, and prolonged transit time.

The present study was undertaken to determine if mice infected with P. berghei would offer a suitable and convenient model for the study of renal pathophysiology. It will be shown that renal dysfunction does occur and appears to be the result of a hemodynamic abnormality.

Methods: Twenty to 30 gm female albino mice were infected with 0.1 ml. of pooled heparinized blood from P. berghei infected mice with approximately a 30% parasitaemia. Haematocrit, parasitaemia, blood urea nitrogen (BUN) and per cent excretion of phenolsulfonephthalein (PSP) were obtained on the 2nd, 4th, 7th, 8th, and 9th days. In addition the kidney was removed for histopathology. Seven animals were studied on day 2, 6 on day 4, 22 on day 7, 25 on day 8 and 9 on day 9. Uninfected control mice were studied by the same methods on each experimental day. On the 11th day, 10 infected and 10 control animals, previously discarded from the above experiments because of PSP dye extravasation at the injection site, were bled to determine BUN and haematocrit. The principles of animal care as promulgated by the National Society for Medical Research were observed.

The procedure was as follows. A 0.25 ml syringe containing PSP was weighed on an analytical balance before and after injection into the tail veins and the exact amount of PSP given was calculated. The dose was usually in the range of 0.02 ml or 0.12 mg of PSP. Pressure was maintained over the injection site for three minutes to insure against loss of dye. Any animal with extravasation of dye was discarded. The mouse was immobilized and a small cup of known weight was appropriately placed to collect faeces-free urine. Sixteen minutes after the Injection of dye the animal was anesthetized with chloroform. Residual urine was washed through the urethra by injection of isotonic saline into the bladder and added to the 16 minute collection. The total volume of urine plus saline was obtained by weighing. The PSP concentration was determined on the total collection. Per cent excretion of PSP was calculated by the following formula:

$$\frac{\text{amount excreted (mg)}}{\text{amount injected (mg)}} \times 100.$$

The urinary PSP concentration was determined by a modification of the method of Rowntree and Geraghty (1912) in the 4th U.S. Army Laboratory Manual (1959). The urine was divided into two aliquots; one was alkalinized with 0.5 N NaOH, the other acidified with 0.5 N HCl. The acidified urine was the blank against which the alkalinized specimen was read at 555 mu on a Beckman spectrophotometer. Pigments, such as hemoglobin, did not interfere with the determination.

Heart blood was drawn for estimation of parasitaemia, haematocrit, and BUN. The number of parasites counted in 1500 red blood cells was expressed as per cent infected red blood cells. The BUN was measured with a Hyland UN Test Kit Method (Hyland Laboratory, Los Angeles, California) utilizing 20 μl of serum.

One kidney from each mouse was fixed in 10% buffered neutral formalin and embedded in paraffin. Sections were cut at seven microns and stained with hematoxylin and eosin. Selected sections were stained by the P.A.S., azure-eosin, and Prussian blue techniques. In addition the liver was removed from mice on the 4th, 7th, 8th and 9th days of study and handled in the same manner as the kidney.

In order to determine the relationship of anaemia to haemoglobinuria ten additional mice were infected at the same time as the previously described animals. Haematocrits from tail blood and urine haemoglobin determinations by the hemacombistix (Ames Company, Inc., Elkhart, Indiana) were performed daily on these animals until death.

Results

Uninfected control animals. The average haematocrit of 35 mice was 51.6 (S.D. · 3.5) per cent. The average BUN of 44 mice was 21 (S.D. · 4) mg per cent. For 35 mice the median per cent excretion of PSP was 20 (range, 12-37 per cent).

Course of infection. Anaemia was first observed on the 4th day of infection and was accompanied by the haemoglobinuria in 2 of 10 animals. During subsequent days as the haemolytic anaemia increased in severity, the haemoglobinuria was marked and was observed in 8 out of 10 animals studied. The haematocrit reached a mean of approximately 20 per cent on the 7th, 8th and 9th days. The parasitaemia rose very rapidly. By the 4th day the mean per cent of red blood cells infected was 55. Though the mean parasitaemia did not change in subsequent days, there were some mice with virtually every red blood cell infected.

Blood urea nitrogen (BUN) (Table I and fig. 1). The mice studied on the 2nd and 4th days of infection had a normal BUN. By the seventh day, however, 15 per cent (3 out of 20) had a mild elevation of BUN. The per cent of animals with an abnormal BUN increased to 67 per cent (6 out of 9) by the 9th day and 80 per cent (8 out of 10) by the 11th. This trend of increasing BUN after day 7 is clearly shown in fig. 1.

Per cent excretion of PSP (Table I and fig. 1). On the 2nd and 4th days of infection the mice had a normal per cent excretion of PSP. Abnormal PSP excretion was observed in 36 per cent (8 of 22 mice) on the 7th day. On the 8th day 56 per cent (14 out of 25), and by the 9th day 78 per cent (7 out of 9) were abnormal. The increased precentage of animals with very low PSP excretion after day 7 was concurrent with the increasing level of BUN (fig. 1). The correlation between BUN and per cent excretion of PSP of individual mice is shown in fig. 2. As the per cent excretion of PSP approached zero the BUN became increasingly elevated. In those mice with zero excretion of PSP the BUN was markedly elevated, with a range of 27-88 mg per cent.

Pathology. Kidney morphology:

Kidneys secured on day 2 resembled normal controls except for mild swelling of the glomerular epithelium and the presence of an occasional parasitized erythrocyte in a glomerular capillary. Kidneys secured on day 4 appeared slightly swollen. The perivascular lymphatics were increased in prominence, containing scattered pigment ladened mononuclear cells.

Periarterial and periarteriolar groups of active appearing reticuloendothelial cells were present in all animals although there was considerable variation in their distribution and number. Kidneys from animals studied on days 7, 8 and 9 appeared markedly swollen, with cortical pallor and variable medullary engorgement. Many of the glomeruli appeared collapsed and bloodless (fig. 3A). The juxtamedullary glomeruli in general appeared to contain more erythocytes than those in the more superficial portions of the cortex (fig. 3B). The basement membrane was not altered. There was no evident necrosis of tubular epithelium, although occasional mitotic figures suggested the possibility of an increase in epithelial turnover rate. The

cortical perivascular lymphatics were dilated and contained large numbers of cells. The intramedullary veins and peritubular capillary plexus showed patchy engorgement (fig. 3C). In many animals the intramedullary portions of Henle's loop also appeared distended. These anatomic changes are believed to suggest significant alteration of cortical blood flow and considerable interstitial edema. An attempt at quantitation of anatomic alteration and correlation with observed physiologic changes was unsuccessful.

Liver morphology:

At 4 days an occasional animal had moderately severe hydropic changes in the cytoplasm of the liver cells surrounding the central vein. In material obtained on days 7, 8, and 9 this change was fairly constant and irregular foci of centrilobular necrosis were also present (fig. 4). No extensive fatty metamorphosis was seen. These lesions probably are related to varying degrees of centrilobular ischemia.

Discussion:

In contrast to the more moderate infection in rats in which renal function was normal (Keeler et al., 1960), the results of this study clearly demonstrated alterations in renal function in P. berghei infected mice. Sadun et al. (1965) found that P. berghei infected mice exhibited an elevation in serum non-protein nitrogen in the absence of an elevated serum creatinine. This would suggest an increased protein catabolism and urea production but not necessarily indicate an abnormality in renal physiology. However, in the present study a low or absent excretion of PSP in those mice with an elevated BUN makes this explanation untenable PSP is excreted mainly by transport across the proximal tubule. Any reduction of blood flow to this area of the kidney or dysfunction of the proximal tubule would lead to a reduction in excretion of this substance. The absence of any tubular pathology would implicate blood flow as the primary abnormality. One other possible explanation for a reduced PSP excretion is a low urine flow due to lower solute excretion or antidiuresis in mice with malaria. This might explain a mild reduction in PSP excretion but would be an unlikely explanation for an excretion of zero. Secondly and more important, the combination of an extremely low PSP excretion and an elevated BUN would reflect a true abnormality in renal function.

The sequence of events leading to azotaemia and renal failure in malaria is unknown. Some mechanism which causes decreased blood flow to the kidney or shunting of blood from the cortex to the medulla, as originally proposed by Maegraith and Findlay (1944), may be involved. The histopathologic findings of a relatively bloodless cortex and congested medullary vessels are compatible with intra-renal shunting of blood, although the dynamic events can not be reconstructed from pathologic examination. A similar pathologic findings has been demonstrated in fatal cases of falciparum malaria (Maegraith and Findlay, 1944) and in other examples of ischemic renal disease (Merrill, 1962). Sitprija et al. (1967) found decreased inulin-PAH clearances in three azotaemic patients with severe falciparum malaria. These patients had no functional evidence of tubular necrosis (low urinary sodium excretion and a high urine osmolality). They felt that in the absence of morphologic changes in the glomerulus or tubular epithelium the best explanation for the azotaemia was an altered renal blood flow. Similarly, Desowitz et al. (1967) found that an elevated BUN and serum creatinine may be found without renal histopathology in the rhesus monkey with P. coatneyi malaria. Undoubtedly the physiologic alterations precede a demonstrable histopathologic lesion. It is believed that on the basis of available evidence the most reasonable dypothesis for renal pathology is a haemodynamic abnormality, although other factors such as toxins or lesions of antigen-antibody complexes must also be considered.

While only renal pathology was considered in this discussion, functional and anatomical changes in other organs, e.g. liver dysfunction with centrilobular necrosis, may well be the result of a similar underlying mechanism(s).

Summary:

(1). Albino mice infected with P. berghei were studied sequentially for changes in BUN, per cent excretion of PSP, renal histopathology, parasitaemia, and haematocrit.

(2). By the 7th day the BUN increased and the per cent excretion of PSP had decreased. These values became progressively more abnormal on subsequent days. In individual mice, as the PSP excretion decreased, the BUN increased. The pathologic abnormality consisted of a bloodless cortex and congested medullary vessels.

(3). On the basis of functional tests and renal histopathology, it is postulated that hemodynamic changes are responsible for the renal disease.

Acknowledgements: We would like to thank LTC D.R. Snyder, M.C. and SFC C. Horton of the clinical laboratory, Clinical Research Center, for performing the biochemicc' determinations.

Legends for figures:

Fig. 1. The per cent distribution of PSP excretion and BUN for mice studied on various days after infection with P. berghei.

Fig. 2. The relationship between BUN and per cent excretion of PSP on days 7,8, and 9.

Fig. 3. Kidney, mouse 135.

A. Collapsed superficial glomerulus.
B. Juxtamedullary glomerulus, erythrocytes seen in capillaries. Malaria pigment present in endothelium.
C. Engorged medullary vessel. Hematoxylin and eosin x 430.

Fig. 4. Liver, mouse 135. Focal necrosis, hydropic degeneration. Hematoxylin and eosin x 250.

TABLE I — The Parasitaemia, Haematocrit, BUN, and Per cent Excretion of PSP on various days of Infection with P. Berghei.

Days After Inoculation with *P. berghei*	2	4	7	8	9	11
Number of Mice	7	6	22	25	9	10
Parasitaemia* (% infected rbc)	8 ± 2	55 ± 9	50 ± 5	52 ± 5	64 ± 4	—
Haematocrit* (%)	52 ± 1	45 ± 2	20 ± 2	18 ± 1	18 ± 1	16 ± 1
BUN** (% mice abnormal)	0	0	15†	41†	67	80
PSP excretion‡ (% mice abnormal)	0	0	36	56	78	—

* The results are expressed as means ± standard error of the mean.

** The mean ± 2SD for BUN in 44 uninfected mice was 21 ± 8 mg per cent. Any determination above 29 mg cent was considered abnormal.

† The number of mice with BUN determination on days 7 and 8 were 20 and 22, respectively.

‡ The range for per cent excretion of PSP in 35 uninfected mice was 12–37 per cent. Any determination below 12 per cent was considered abnormal.

Fig. 1. BUN and PSP excretion in P. berghei infected mice.

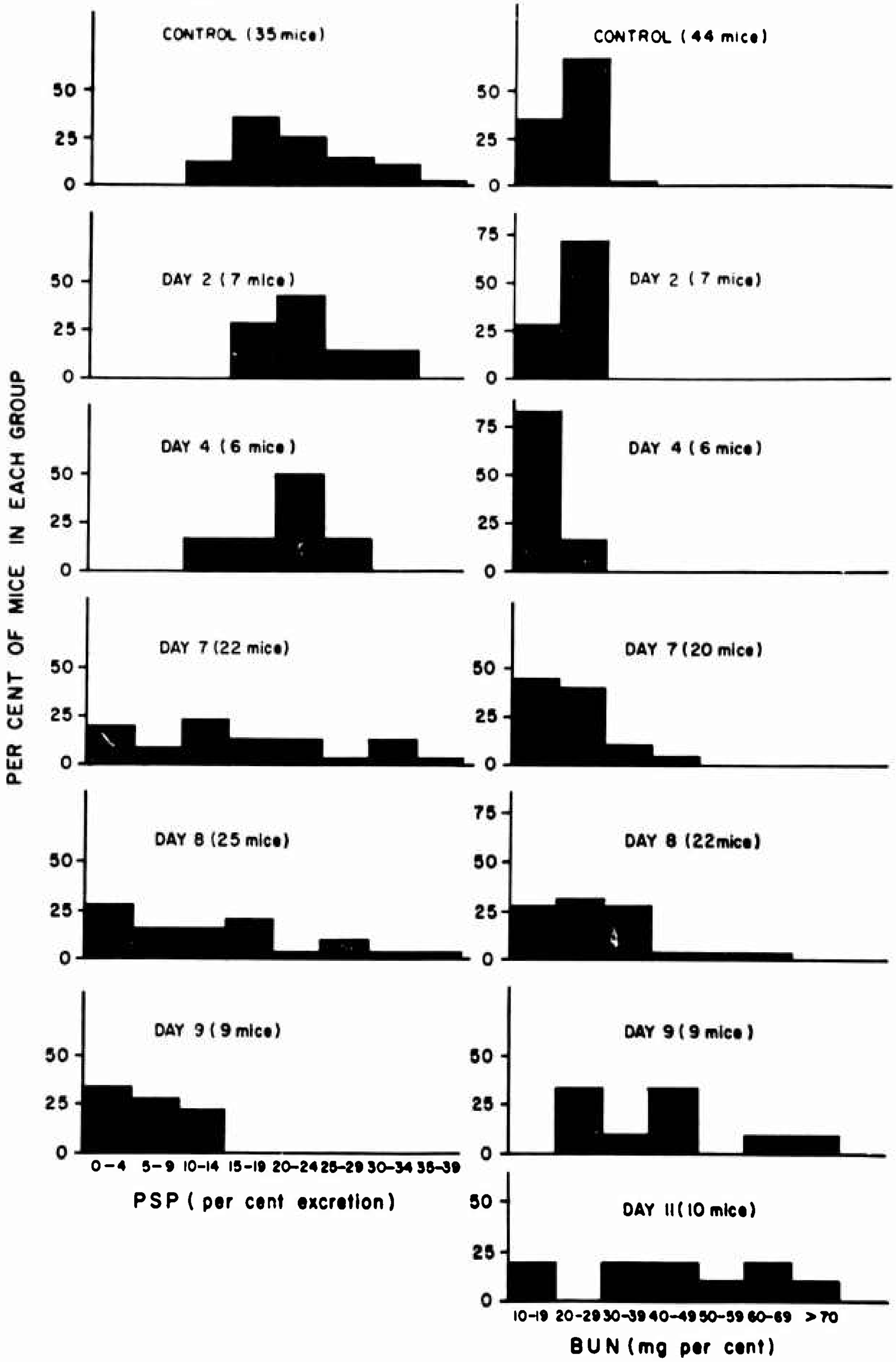


Fig. 2. Correlation between BUN and PSP excretion in P. berghei infected mice

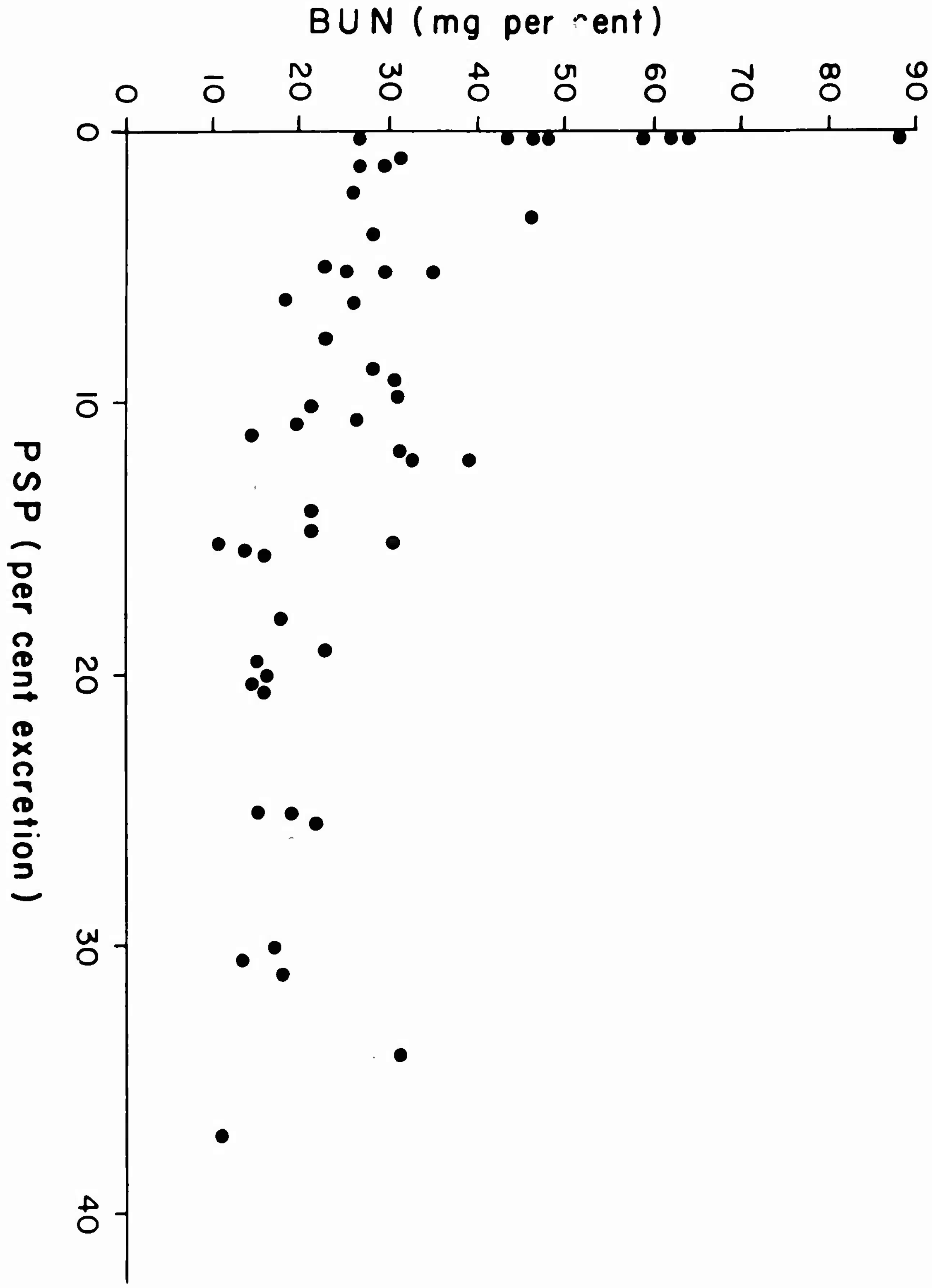


Fig. 3a. Histopathology of the kidney of P. berghei mice.

Fig. 3b. as Fig. 3a.

Fig. 3c. same title as Fig. 3a

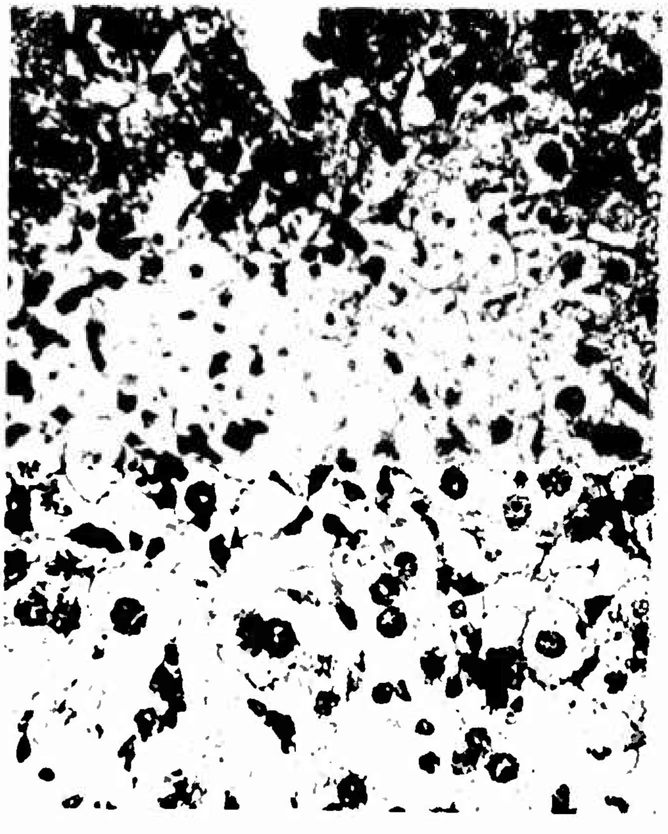


Fig. 4. The liver of a P. berghei infected mouse showing centrilobular necrosis

Subtitle: Immunization of Rats Against Plasmodium Berghei with Plasmodial Homogenate, Carboxymethyl-cellulose (CMC) Bound Homogenate, and CMC-Homogenate Followed by Administration of "Immune" Gamma Globulin

Investigator: R.S. Desowitz

The possibility of producing effective active immunization against malaria has occuppied the attention of many investigators for more than fifty years. While there is no single recent comprehensive review of the many attempts to induce a protective immunity, reference can be made to the pertinent papers in the Proceedings of the International Panel Workshops (1964, 1966).

Most attempts at immunization have employed killed plasmodia. The success with this form of antigen has, in general, been highly variable but this is not suprising considering the many different methods of antigen preparation, immunizing schedules, species of Plasmodium and hosts used. There is indication that immunization with adjuvant-antigen mixtures afford better protection than antigen alone (Freund et al, 1945, 1947, 1948). Successful immunization of monkeys was obtained by Targett and Fulton (1965) by use of P. knowlesi-Freunds adjuvant. Intramuscular injection of the mixture seemed to obviate the untoward effect usually attendant upon the use of Freund's adjuvant. Zuckerman et al (1965) noted a partial protection of rats against P. berghei after they had been given a series of immunizing doses with cell-free homogenates of parasites. These authors found that the only difference between inoculation with or without adjuvant was that a single does of the adjuvant-antigen was apparently as effective as three doses of the antigen alone.

Moroz and her colleagues showed (1963) that the immunogenicity of viper venom neurotoxin was enhanced when bound to soluble carboxymethly-cellulose (CMC). This promising technique had not been applied to parasite immunology and it was thought of interest to determine whether a similar effect could be could be obtained with malaria homogenate bound to CMC. This present communication gives the results of P. berghei homogenate CMC immunization and the effect of "immune" gamma globulin given to antigen-CMC immunized rats prior to challenge.

Methods

White rats weighing between 200 and 225 grams were used in these experiments. The advantage of using the white rat is that unlike the mouse, fulminating infections are not generally produced, thus any partial immunizing effect is more likely to be detected.

The principles of laboratory animal care as promulgated by the National Society for Medical Research were observed.

Antigen was prepared from the pooled blood of heavily infected mice. Parasites were freed from the erythrocytes by the dextran-saponin method of Spira and Zuckerman (1966). Homogenate of the washed plasmodia was then made by use of a Hughes press. Micro-Kjeldal analysis was performed for all antigen preparations which were then diluted in 0.005M phosphate buffer (pH6.8) to contain between 6.5 to 7.5 mg protein per ml. The extract was divided in half and to one portion was added 40 mg CMC per ml according to the method of Moroz et al (1963).

In the first experiment, of 60 rats one group of twenty was inoculated with 0.1 ml antigen intramuscularly and another group of 20 given 0.1 ml antigen-CMC by the same route. A group of 20 rats were set aside as controls. Three immunizing doses spaced two weeks apart were given. Two weeks after the last inoculation all animals, including the 20 controls, were challenged by intraperitoneal inoculation with approximately 68 x 10^6 parasites in 0.1 ml mouse blood. Thin blood films of each animal were made daily

for thirty consecutive days. These films, made by an experienced technician were with of a high degree of uniformity. The assessment of immunizing effect generally followed that used by Zuckerman et al (1965). The total number of parasites in 100 thin oil-immersion fields were counted and from this the first day of patency, the number of plasmodia at peak parasitaemia and the day at which peak parasitaemia occurred were noted. While it is recognized that there are inherent deficiencies in this method of estimating parasitaemia it is probably no less accurate than more complicated enumerative techniques. Given slides prepared with reasonable uniformity it is felt that this method gives an acceptable means of assessing the course of the parasitaemia.

At the end of experiment one, all the rats were reinoculated with 0.1 ml heavily infected mouse blood and killed two weeks later. The globulin from the pooled sera of all sixty animals saturated ammonium sulfate method of Kendall described by Kabat and Mayer (1964). Paper electrophoresis showed that the fraction so obtained was almost entirely gamma globulin with a small amount of beta globulin.

In the second experiment, of forty white rats, twenty were immunized with CMC-antigen as in the first experiment. Two days prior to challenge these animals and ten non-immunized rats were intraperitoneally inoculated with approximately 15 mg of gamma globulin on each of two successive days. These rats along with ten controls were then challenged with approximately the same number of parasites used in experiment one and the course of infection studied exactly as in that experiment.

Since both the antigen and challenging parasites were derived from mouse blood the possibility existed that some of the immunizing effect might be due to antibody produced to mouse erythrocyte material. In order to determine whether this factor was present 20 rats were immunized with stroma prepared from uninfected mouse blood. This material was obtained in the same manner as that for an equal amount of infected erythorcytes. When these rats were challenged following the completion of the immunizing course it was found that the prepatent period was extended for an average of 0.5 days as compared to the controls. The average peak parasitaemia attained by both groups was not significantly different.

Results

The results are summarized in Table 1. Since Students t-Test showed no significant difference in parasitaemias between the control groups of experiments one and two all results are listed together for convenience of comparison. Both antigen alone and antigen-CMC produced an immunizing effect. However the degree of immunity produced by antigen-CMC seemed to be of a higher other than antigen alone. The average parasitaemia of the antigen group was approximately one half that of the controls and that of antigen-CMC one third. The number of days of prepatency and peak parasitaemia of both immunized groups were greater as compared to the controls. The median and mode of the prepatent period for the antigen-CMC group was slightly longer (4 days) than that of the antigen group (3 days).

The inoculation of the "immune" gamma globulin had no effect on the intensity of parasitaemia ultimately produced. However, the parasitaemia of those rats given antibody developed more slowly, as reflected by median-mode days of peak parasitaemia than that of the controls. That there was a combined effect of active immunization followed by passive transfer of antibody was evidenced by the considerably longer median-mode prepatent period (7 days) and day of peak parasitaemia (10.9 days) of the antigen-CMC-gamma globulin group than of the group immunized with antigen-CMC. While the average peak parasitaemia, was the same for both groups it is of interest to note that only in the group given antigen-CMC-gamma globulin were there any rats (3/20) completely protected for the entire 30 day observation period.

During the thirty day observation period, the rats in all groups experienced a number of parasitaemic recrudescences which were generally of a progressively diminishing intensity. There is some evidence, despite a wide variation in parasite densities from rat to rat within any one group, that immunization affected the intensity of the parasitaemia at least at the first recrudescence. In experiment one

the average density at the first recrudescence for the controls was 753/100 thin oil-immersion fields, whereas for the group immunized with extract only it was 479 and for the group given extract-CMC it was 290. There was little difference in the nature of the first recrudescence between the groups given antigen-CMC and antigen-CMC-gamma globulin.

Discussion

The results presented in this paper indicate that immunization with antigen bound to CMC produced a significantly superior immunity to that of antigen alone. That the homogenate antigen also gave some partial protection by lowering the parasitaemia and extending the prepatent period is in agreement with the findings of Zuckerman et al (1965). In contrast to the use of antigen-CMC, these authors reported that there was no advantage to the use antigen-Fround's adjuvant other than shortening the course of immunization. Immunization with antigen-CMC seems to be worthy of further trial and experiments with primate malarias are planned.

That administration of antibody alone slowed the progress of the parasitaemias but did not ultimately effect the ultimate course of infection confirms the recent work of Briggs et al (1966). There was an additive effect of active immunization plus "immune" gamma globulin in that the prepatent period was considerably increased by this treatment. It is felt that this line of investigation also deserves further study using larger amounts of gamma-globulin and with other host-malaria parasite systems.

Table 1. The effect of immunization with P. berghei extract, extract–CMC, and extract–CMC followed by administration of "immune" gamma globulin.

Group	1st Day Parasitaemia			Peak Day Parasitaemia			Peak Parasitaemia	
	Median	Mode	Range	Median	Mode	Range	Arith. mean	95% Confidence Limits
Controls n = 30	1	1	1–7	5	5	1–9	1,230	1,050–1,410
Gamma globulin n = 10	1	1	1–6	8	8	6–10	1,473	1,222–1,724
Antigen n = 20	3	3	2–8	7	6	5–12	635	542–728
Antigen – CMC n = 20	4	4	2–7	7	7	6–12	403	323–483
Antigen – CMC gamma globulin n = 20	7	7	4–00*	10	9	8–100*	440	335–545

* Three animals did not develope parasitaemia during the 30 days followed.

Title: Nonhuman Primate Malarias in Thailand

Principal Investigators: Robert S. Desowitz
Katchrinnee Pavanand

Assistant Investigator: Barnyen Permpanich

Objective:

There are few, if any, records of primate malarias in Thailand. The discovery of plasmodia in monkeys and gibbons in Thailand is of interest not only because of possible transmission to man but also because comprehensive studies on newly isolated strains may illuminate some of the problems related to human malaria. Furthermore, the presence of primate malaria in an area may complicate identification of the vector of human malaria in that same area.

Description:

Blood films of all primates purchased by the Veterinary Department are sent to the Parasitology Department for routine examination. To date 124 blood films from wild, caught cynomolgus and irus monkeys have been examined. Of these 18 (14.5%) have found to be infected. In all cases the parasite was tentatively identified as P. inui. It would thus appear that the infection rate may be high. It has been shown (Ann. Rep., 1966) that the Thai strain of P. inui is reabily transmitted by Anopheles balabacensis. Since this mosquito is also implicated as a major vector of human malaria it would be of importance to identify the species of sporozoite in infected wild-caught mosquitoes.

One hundred and seventy four slides from wild-caught gibbons have been examined and 2 (1.14%) were found to be positive. One infection was from a gibbon that came from the area near Chumporn in S. Thailand. The strain is being maintained in the laboratory and has been identified as P. jefferyi.

The other isolate came from Trat, N. Thailand and is also being maintained in gibbons. Identification of this parasite has proved most difficult since during the course of infection it shows morphologic characteristics similar to all four plasmodia described as natural gibbon infections; P. jefferyi, P. eylesi, P. youngi, and P, hylobati. Dr. McWilson Warren of the NIH and ourselves were first of the opinion that it was a double infection of P. eylesi and P. jefferyi. Prof. Garnham thought it a double infection of P. eylesi and, because of some small gametocytes, P. hylodati (a parasite described only once by Rhodain some 20 years ago). However, careful sequential examination during the course of infection has revealed peculiar transitional forms and so the possibility of a remarkably pleomorphic parasite cannot be entirely ruled out. Eylesi forms with stippling and multiply infected erythrocytes appear early in the infection and gradually give way to predominantly jefferyi and youngi-hylobati types.

The morphology and schizogonic cycle was studied in detail in P16, the second blood subpassage, from the 79th to 86th days of infection. Thin blood films were taken at 4-hourly intervals during this entire period and from this the periodicity and morphology studied. At least 100 parasites, taken at random, were drawn from each blood film. Photographs were made of typical parasites at different growth stages and then drawn in semi-stylized fashion from projections of color slides (Plate 1).

Only parasites resembling P. jefferyi were observed to be present at this time. While the morphology generally conformed to the description of Warren, Coatney and Skinner (J. Parasit. 1966, 52)

certain differences were observed. Firstly, double infections were seen in about 2%-3% of all parasitized erythrocytes (Fig. 3) and secondly, true stippling of the infected erythrocytes was absent at all stages. Furthermore, it is our present belief that the parasite may be pleomorphic and growth may proceed through somewhat different forms. The most typical sequence seems to be as follows: The merezoite enters the erythrocyte (Figs 1 & 2) and a fine ring with a prominent single compact nucleus develops (Fig 3). The ring enlarges (Fig. 6) and amasses cytoplasm (Figs 7, 8, 11). These older trophozoites are not amoeboid and accumulate a find dust-like golden pigment along their periphery. There is a large vacuole. The parasite occupies the entire erythrocyte, often destroying and growing beyond the original rbc boundary (Figs 17, 18, 19). At this stage the nucleus becomes band-like preparatory to division. The next stage, proceeding to the pre-schizont and early schizont, involves a disappearance of the vacuole and consolidation of the cytoplasm (Figs 21-25). Typically, this form is seen to occupy about half the space of that of the mature trophozoite and only the outline or ghost of the original, now destroyed erythrocyte cell wall is evident. The pigment may be either finely particulate or clumped in prominent golden-colored masses.

A variation in development is that the ring, early in development, shows a tendency toward amoeboidicity and irregularity. This amoeboid behaviour is apparrent throughout its progessive development even to the schizont which has wispy strands of cytoplasm rather than being compact. The successive stages of this type of development are shown in Figs 12, 13, 14, 15, 16 and 28.

A second growth variation is that the rather huge mature trophozoite stage (Fig. 18) may be by-passed. The progression of events here is that the small ring rapidly acquires both cytoplasm and pigment (Fig. 4). It grows to the late and mature trophozoite occupying about half or two thirds of the erythrocyte and without destroying it (the remaining rbc material appears to be normal). Finally, the schizont of this "miniature" form also occupies only a portion of an otherwise normal appearing erythrocyte (Figs 26, 27).

A rather unusual form of growth is shown in Figs 10 and 16 and 20. The compact nucleus is within the center of the growing ring. The trophozoite amasses cytoplasm and the nucleus undergoes a pre-divisional development until the form seen in fig 20 is attained.

The points which lead us to the conclusion (tentative) that these are all morphologic variations of one species are: (1) There is no anisocytosis of the host erythrocyte nor is it stippled (enlarged parasitized rbc are seen but there is a severe anemia with this infection and at times as many as 20% or more of the erythrocytes are reticulocytes). (2) The pigment in all developmental types is of a fine dust-like golden appearance consolidating into clumps at later stages. (3) Schizonts of all types are rare in the peripheral blood and development is usually completed in the deep circulation system.

The periodicity is shown in Fig. 31. Since schizongony is not usually completed in the peripheral circulation the best evidence of periodic behavior is the percentage of very young rings found. There appears to be a triple brood of parasites (a double brood was noted in the gibbon studied by Warren et al). There is a distinct main brood exhibiting regular tertian periodicity. The main peak of these small ring forms appear of quite regularly at 1100 to 1500 and the other peaks occurred 0300 and 1500) on alternate days to the main brood.

Fig. 1. Morphologic variation in development of parasites from Gibbon P 16

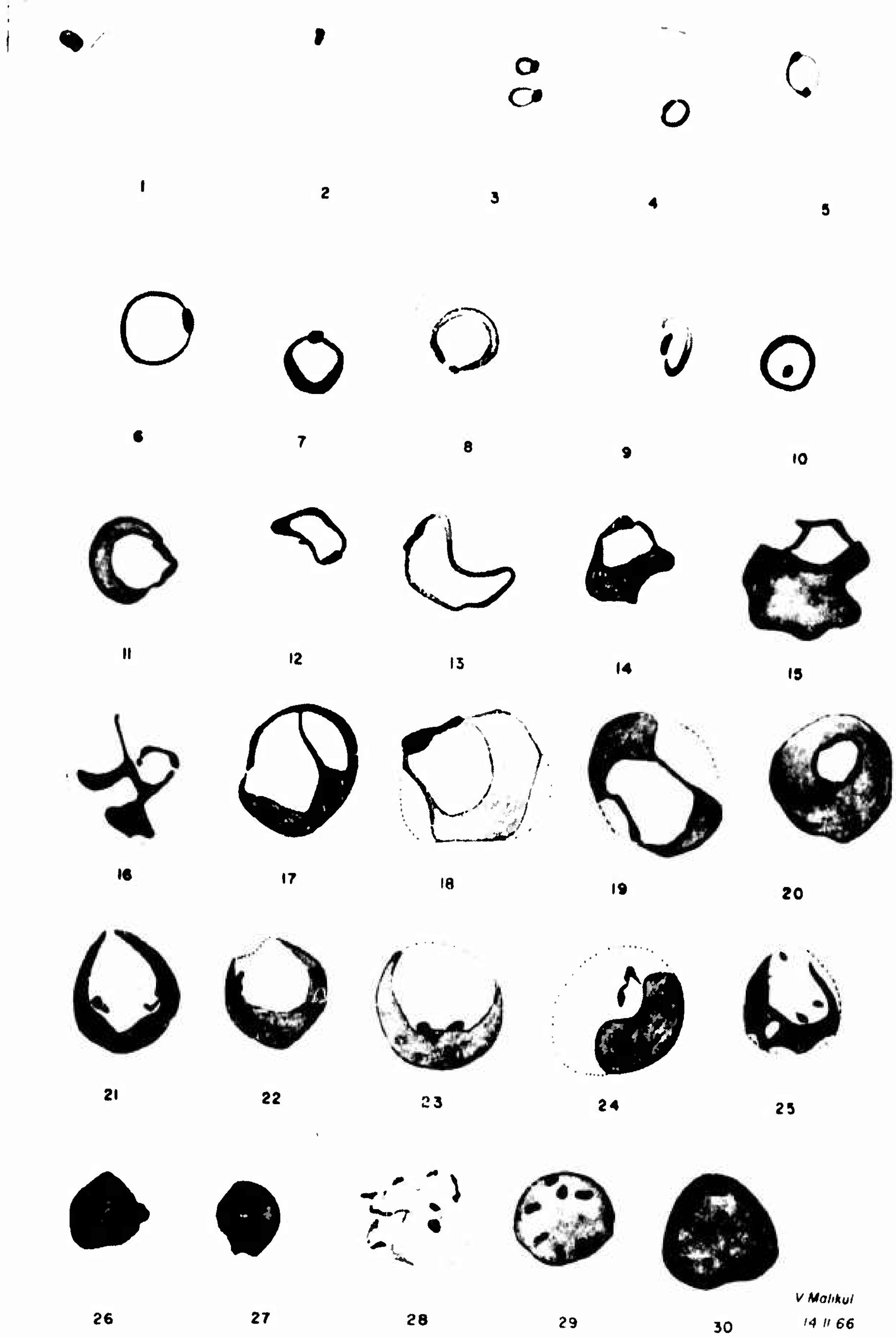
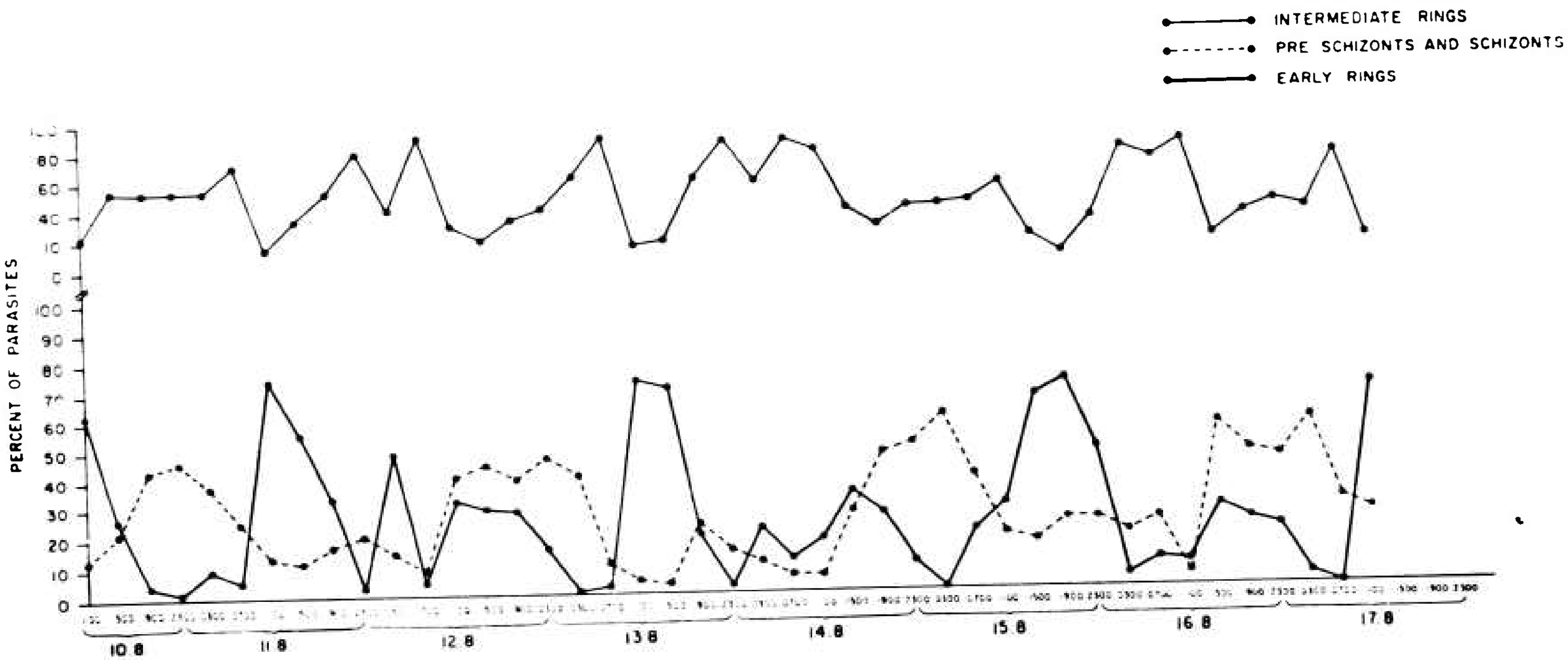


Fig.2.GIBBON P-16.

DIFFERENTIAL PARASITE COUNTS MADE AT 4-HR INTERVALS



Title: THE PHYSIOLOGY OF THE MALARIAL PARASITE

Principal Investigators: Bernhardt W. Langer, Jr., Ph.D.
Robert S. Desowitz, Ph.D., D.Sc.

Associate Investigator: Pirom Phisphumvidhi, B.S.

Assistant Investigators: MSG Yael Friedlander
SFC Harvey Gibson

Objective:

It is the purpose of this study to investigate in detail the anabolic and catabolic metabolism of rodent, simian, and human malarial parasites. Certain aspects of host metabolism, particularly those which bear directly on the host-parasite relationship, are also being examined.

Methods:

The metabolic pathways of the parasite and host cells are being examined using combinations of isotopic, spectrophotometric, and respirometric methodologies.

Progress:

The activities of glucose-6-phosphate dehydrogenase (G6PD), 6-phospho-gluconate dehydrogenase (6PGD), and tranketolase were studied in cell-free extracts of normal and Plasmodium berghei-infected mouse erythrocytes and host cell-free P. berghei. All preparations had activity. Electrophoretic evidence clearly indicate that the G6PD and 6PGD activities were of P. berghei origin. The high specific activity of transketolase in the P. berghei extract as compared to the other preparations showed that it was also of parasitic origin. The presence of phosphoriboisomerase and ribulose phosphate-3-epimerase were indirectly demonstrated. The data clearly support the conclusion that the malaria parasite, P. berghei has an active pentose cycle. A publication, in press, entitled "Malarial Parasite Metabolism: I. The Pentose Cycle in Plasmodium berghes." has resulted from this work.

Recent efforts have centered on the use of the French Pressure cell as a means of selectively rupturing the infected erythrocyte in hope of improving the quality of the isolated parasitic preparation. The data at present, are insufficient to permit any detailed conclusions to be drawn.

Methods for the investigation of glycolysis and the tricarboxylic acid cycle are in the process of being developed and on results are available at this time.

Studies on the metabolism of the simian and human parasites are still in the preliminary stages with no data available.

Summary:

A pentose cycle has been demonstrate in the rodent malarial parasite, Plasmodium berghei. Development of methodologies for further studies of the metabolism of P. berghei. P. coatneyi, and P. falciparum is currently in progress.

Title: Transmission of Plasmodia to Heterologous Hosts

Principal Investigators: Francis C. Cadigan, Major, MC
Robert S. Desowitz, Ph.D., D.Sc.
Katchrinnee Pavanand, M.D.

Species of the genus Plasmodium have been considered to be highly restrictive in their host parasite relationships. There is, however, a growing body of evidence that some plasmodia may infect heterologous hosts. The ultimate aim is to adapt the human malarias to a convenient laboratory animal such as the white rat or mouse. In addition, the processes by which plasmodia may be induced to infect normally non-susceptible hosts can give valuable information on the dynamics of host parasite relationships.

This report summarizes the results of a number of experiments on experimental transmission of primate malarias to heterologous hosts.

1. Attempts to infect the white rat with primate malarias. (K. Pavanand and R.S. Desowitz)

a. P. inui: Three splenectomized and two intact young adult rats (weight: 50-70 gm.) were inoculated via the tail vein with a washed saline suspension of erythrocytes infected with P. inui (484/10,000 rbc). Blood smears were made daily for five days and weekly thereafter. One splenectomized rat showed ring forms 48 hours after inoculation and was negative thereafter. All other animals remained negative.

b. P. coatneyi in immuno-suppressively treated rats: Two rats, one splenectomized and one intact, were given per os 5-7 mg./kg of Purinethol daily for 6 days. The course was begun two days before being infected with P. coatneyi via the tail vein. Two other untreated animals (one splenectomized and one intact) were inoculated with the infected monkey blood at the same time. No parasites were found in any of the rats during the one month that blood films were examined.

c. Gibbon malaria:

The first trial employed four adult rats. One splenectomized and one intact rat were treated with Purinethol as described in the preceding experiment. One intact and one splenectomized rat were left untreated. All animals were intravenously inoculated with heavily infected gibbon blood (parasitaemia 1086/10,000 rbc). Blood smears were taken daily. The intact, unreated rat showed pigment in the leukocytes three days after inoculation but no parasites were detectable. On the 6th day many well-developed ring forms were seen. The blood was negative thereafter. All other animals were negative throughout the two week period of observation.

The second experiment employed younger rats weighing between 40-60 gm. Immunosuppressive was not given in this trial. Five rats were splenectomized and four remained intact. Blood from gibbon P-9 (parasitaemia 876/1000 rbc) was washed twice, resuspended in sterile physiologic saline and 0.7-1.5 ml. and inoculated via the tail vein. All rats showed parasites in the blood up to 48 hrs after inoculation. In one rat (intact) a single ring form was again seen on the 21st day.

The third series of trials employed newborn rats not older than 24 hrs. Ten newborn rats were inoculated with 0.50 ml. of heavily infected blood from gibbon P-9 (parasitaemia 1004/10,000 rbc). Parasites were seen in blood films made 28 and 93 hrs. after inoculation. On the 13th day three animals remained alive and in two of these what appeared to be very young pre-ring stage forms (chromatin dot

with small solid cytoplasmic appendage, merezoite-like in appearance and size) were seen. The infections in these animals were very scanty. That these forms were probably parasites and not artifacts was indicated by their absence in ten newborn control rats inoculated with the blood of an uninfected gibbon.

A second group of 11 newborn rats were inoculated intraperitoneally with blood from gibbon P-9 (parasitaemia 618/10,000 rbc). One animal showed parasites in the peripheral smear within 16 hrs. and all others at 24 hrs. Nearly mature schizonts resembling P. jefferyi were seen in the film from one animal at 93 hrs. and in another at 141 hrs. Three animals were sacrificed 5 days after inoculation and the pooled blood inoculated into 6 newborn rats. Twenty-four hours after inoculation, two rats were dead, smears made from heart blood of one rat revealed mature schizonts. Daily blood films from the remaining rats were negative until the 9th day when very scantly young forms resembling merezoites were seen in all animals. One erythrocyte was found to be doubly infected with these forms. No parasites were seen after the 9th day. One of these rats was sacrificed on the 9th day and its blood inoculated into 5 newborn rats. Four of those rats (2nd subpassage) showed a scanty parasitaemia of young pre-ring forms 17 hrs. after inoculation. In one rat a developing trophozoite was seen on the 4th day.

These subpassages in new born rats are shown schematically below.

Gibbon P9
6 Days
1st gibbon to rat passage
5 days
1st rat to rat subpassage
9 days
2nd rat to rat subpassage
(positive 4th day)
24 days total sojurn in rat host

These results of these experiments indicate that it might be possible to adapt gibbon malaria (probably P. jefferyi) to the newborn rat. While the survival time of gibbon erythrocytes in the rat is as yet unknown, the finding of parasites 24 days and two subpassages later would make this factor unlikely, at least at the later subpassages. The presence of what appeared to be very young forms is evidence that at least one schizogonic cycle had occurred. The reason for the better success with gibbon malaria as compared to monkey malarias is as yet unknown. However, preliminary experiments have shown a greater compatability between rat and gibbon bloods than between rat and monkey.

2. Primates as heterologous hosts. (R.S. Desowitz and F. Cadigan). These experiments have two objectives: 1. To determine the biologic relationships between the plasmodia of higher and lower primates. 2. To determine if infection with one might provide some immunologic protection against another, particularly between apparrently related species such as P. coatneyi and P. falciparum, or P. inui and P. malariae.

a. Attempts to infect gibbons with P. coatneyi. Preliminary observations on the cross-immunity between the two species.

Two gibbons were inoculated intravenously with 2 ml. blood from a P. coatneyi infected rhesus. One gibbon S9 had not been experimentally infected previously and was considered "clean" while gibbon P9 had been imfected with P. falciparum although the animal no longer had parasites in the peripheral blood films. Thirty-six days after inoculation a scanty parasitaemia was seen in gibbon S9. The parasitaemia continued at this low level for two weeks and then disappeared. One month after the last positive blood film the animal was inoculated with blood from the "gibbon line" of P. falciparum. The animal became infected with a course of parasitaemia no different than "non-immune" controls.

P9 showed a P. falciparum infection for 44 weeks. Approximately 6 months after the last positive blood film it was challenged with P. coatneyi. This animal did not subsequently become infected.

Further trials on cross immunity engendered by these two plasmodia are now in progress.

b. Attempts to infect rhesus monkeys with gibbon malaria: Two rhesus monkeys were inoculated intravenously with blood from a heavily infected gibbon. Neither became infected.

c. A comparison of the infection following inoculation of gibbon malaria into animals previously infected with P. vivax and P. falciparum with animals not previously inoculated revealed no evidence of any degree of protection afforded by either P. vivax or P. falciparum against the gibbon malaria.

d. To determine how specific is the susceptibility of gibbons to mala[illegible], several splenectomized white-capped gibbons (Hylobates lar pileatus) have been obtained and inoculated with P. vivax and P. falciparum. Results thus far indicate that this sub-species is far less susceptible than Hylobates lar lar at least insofar as patent peripheral parasitemia is concerned. No conclusions can be made as this study has been in progress for only a short time.

Title: Haemosporidiidea of Thai Birds

Principal Investigator: R.S. Desowitz, Ph.D., D.Sc.

Associate Investigators, Chanpen Punyarcung
E. McClure
B. King
J. Marshall

Objective-In the course of their investigations the MAPS and Virus Department have collected many thousands of blood smears from wild animals, primarily birds. It is of interest to determine the types of blood parasites infecting these animals. It might be of specia importance if any particular group of birds had a high percentage of plasmodial infections. It could be inferred that there is preferrential mosquito feeding on that group and because of this they might also have a particularly high rate of mosquitoborne virus infections.

Progress - Because of the very large numbers of slides involved the first phase has been a preliminary screening for blood parasites. At this stage only generic diagnosis is made. In this manner over 5,000 slides have been examined to date. The positive slides have put aside for future study here or by specialists in avian blood sporozoa. Analyses of infection according to host species and region are in progress but has not been completed.

Results: Total slides examined 5,344

No. positive Haemoproteus	789 (14.7%)
No. positive Plasmodium	8 (0.14%)

It is obvious that the greatly predominant infection of birds is Haemoproteus. Very little plarmodia has been found.

Title: Anopheles and Malaria

Principal Investigators: Douglas J. Gould, Ph. D.
Louis C. Rutledge, Capt., MSC

Assistant Investigator: Sahem Esah

Objective.

The objective of this study is the investigation of those species of Anopheles responsible for the transmission of human malaria in Southeast Asia. Studies on the bionomics and population dynamics of anophelines have been undertaken in malarious regions of Thailand. Fluctuations in the density, species and age composition of anopheline populations are being measured in an effort to relate these factors to the incidence of malarial infections in both the human and mosquito populations. Specific factors being studied in the attempted definition of potential vector species in Thailand also include the determination of their flight range, longevity, patterns of biting activity, host preferences and susceptibility to infection with the plasmodia causing human malaria.

Description.

Important regional differences exist in Thailand with respect to the incidence of malaria and the species of Anopheles serving as vectors. At present, approximately 52 species of Anopheles have been recorded from this country, and about half of these species are known to feed on human beings. Anopheles minimus is responsible for much of the malaria occuring in the foothills of this country, and A. balabacensis has been recognized as an important vector species in many of the forested parts of Thailand. The vector status of other anophelines in Thailand, such as A. maculatus, A. sundaicus, A. campestris, A. aconitus and A. philippinensis, which are primary vectors of malaria in other parts of Southeast Asia, remains to be clarified. Permanent or recurrent studies of the anopheline populations in Nakornrajisima, Chantaburi, Phatumthani, Narathiwas, Songkla and Satun provinces have been established in areas representative of the ecological associations existing in malarious regions of Thailand.

Phatumthani Studies The central plains of Thailand surrounding Bangkok have until recently been considered free of all but introduced cases of malaria, and this region has not been included in the residual spray program of the national malaria eradication effort. In recent years it has become apparent that foci of low level malaria do exist in this region which have certain peculiarities distinguishing them from other malarious areas of Thailand 1) none of the proven vector species, such as Anopheles minimus or Anopheles balabacensis, occur there, 2) the majority of the malaria infections are caused by Plasmodium vivax rather than P. falciparum and 3) the peak in incidence is reported to occur in the dry season (January-May) rather than in the rainy season. Thus, in February 1960 in Nontaburi province the National Malaria Eradication Project reported that 3.4 per cent of 3830 persons examined were infected with P. vivax while another 0.3 per cent had P. falciparum infections. Anopheles campestris was the commonest anopheline caught in houses at the time, but the results of dissections of over 2000 females of that species were negative.

During April, August and November 1966 entomological surveys were carried out by SMRL in a further attempt to define the vector (s) of malaria in the Central Plain area. Malaria surveillance reports

indicated the presence of malaria in villages of Sam Kok district in Phathumthani province adjacent to the Chao Phrya river some 20 miles north of Bangkok. These villages extend alongside the river bank for as much as a kilometer or more but are no more than one or two houses deep at any point. Gardens and rice fields tended by the villagers are located along the backside of the villages. Examination of thick blood smears from 615 Sam Kok villagers in April showed 22 (3.6%) P. vivax infections. No cases of falciparum malaria were found.

Biting collections of anophelines were made at Sam Kok between 1900 and 2400 hours using human bait. Six species A. aconitus A. annularis, A. campestris, A. philippinensis, A. tesselatus and A. vagus were predominant in these collections. Anopheles campestris was the species most frequently caught indoors, while the other five species were collected with significantly greater frequency outside houses than indoors. The results of dissection of approximately 3000 anophelines collected during this period are shown in table 1.

Table 1. Results of salivary gland and gut dissections of anopelines collected in Phatumthani Province-1966.

Species	Number infected/Number Dissected		
	April	August	November
Anopheles aconitus	1/18	1/81	0/374
Anopheles annularis	0/3	0/57	0/70
Anopheles campestris	0/301	0/59	0/601
Anopheles philippinensis	0/15	0/218	0/181
Anopheles tesselatus	0/221	0/62	0/374
Anopheles vagus	0/139	0/77	-

The specimen of A. aconitus found infected during April had both sporozoites and oocysts and had partially fed upon the collector when captured. Eight days later the collector complained of headache and fever (102°F), but no parasites were seen in a thick blood smear taken on that day. On the 20th day following his exposure this man had a topical paroxysm, and trophozoites and schizonts of P. vivax were observed in his blood. A second infected specimen of A. aconitus collected in August had oocysts but no sporozoites. The above circumstances strongly suggest that A. aconitus is one of species involved in malaria transmission in the Central Plain of Thailand. This mosquito has not been previously implicated in Thailand, but it has been considered an important vector in parts of Indonesia and Viet Nam.

Previous studies in the Pak Chong valley area, where dwellings have been sprayed with DDT for almost a decade as part of malaria eradication activities, demonstrated that A. minimus, A. maculatus and A. aconitus man-biting rates were consistantly higher outdoors than indoors. The question arises as to whether or not these differences represent adaptive changes on the part of these anophelines in response to the insecticide pressures exerted in that area. Because there have been no such pressures applied to the anopheline populations in the central plain area a similar comparison of indoor-outdoor biting rates was made at Phatumthani. Unfortunately, neither A. minimus nor A. maculatus are present in this region, but the observed biting rates for A. aconitus. A. phillipinensis, A. tesselatus and A. vagus were significantly

higher outdoors than indoors in Phatumthani (Table 2). On the other hand, A. campestris showed no such reluctance to enter and feed on the occupants of houses. These observations suggest that the exophilic behavior of species such as A. aconitus is inherent, and that outdoor transmission of malaria must be suspected where such anophelines are important vectors (see section below on biting activities of A. balabacensis at Sadao). In such situations malaria eradication efforts which rely solely upon application of residual spray to the interior of dwellings are not likely to interrupt transmission.

Table 2. Observed differences in numbers of anophelines collected biting indoors and outdoors in Phatumthani Province, 1966-67.

Species	November, 1966		January March, 1967		Level of significance
	Indoors	Outdoors	Indoors	Outdoors	
A. aconitus	142	931	27	478	1+
A. campestris	269	254	37	58	n.s.
A. phillipinensis	127	593	0	9	1+
A. tesselatus	57	176	16	411	1+
A. vagus	8	30	0	23	1+

Sadao Studies-The Malayan border area has been the subject of several previous surveys by SMRL because the identity of the vector species in that region is not known with certainty. Anopheles minimus is known to be present only in Satun province. Previous studies in Narathiwas Province (1964, 1965) yielded some evidence of malarial infections in A. karwari and A. maculatus. Evidence on the vector status of A. balabacensis in these areas was negative, as efforts to collect adults of this species on previous trips were fruitless. During June entomological and malaria surveys were made in Sadao district, Songkla Province, at the village of Ban Khao Roop Chang, near the Thai-Malayan border. Thick blood films from 171 villagers at Ban Khao Roop Chang were examined by personnel of the Department of Epidemiology on 13 June, and 43 (25+) were positive for P. falciparum, 10 (6+) for P. vivax and 1 (0.6+) slide demonstrated parasites of P. malariae. Biting collections were made on 13 nights between the 1800-2400 hours. The results of these collections are summarized in Table 3. On two nights collectors were also placed inside village houses, but no anophelines were collected biting indoors. Attempts to find resting anophelines during early morning hours (0600-1000) in village huts were also negative. Three of 202 A. balabacensis dissected during this period were found infected with malarial parasites one with sporozoites in the glands, one with infected glands and oocysts and a third with oocysts only. This mosquito was the dominant man-biting species collected during these studies. Ovarian dissections revealed that 80 per cent of the balabacensis were parous, and that 10 per cent of the parous females had two or more previous blood meals. Approximately 14 per cent of the parous females collected were in a gravid or pregravid condition, and it was impossible to determine the number of dilatations present. The occurence of such a substantial number of gravid females in these biting collections suggests that the taking of a second blood meal during the course of an ovarian cycle may occur with significant frequency. The vector potential of such females is of course much greater. In contrast the other species of anopheles especially A. philippinensis had a

higher percentage of nulliparous females than did balabacensis indicating that the daily survival rate of these species was significantly lower than that of balabacensis. Furthermore, no infected mosquitoes were found among these other species. The pattern of biting activity (Table 4) exhibited by balabacensis during the Sadao studies was also remarkable in that the peak occurred between 1900 and 2000 hours while most of the villagers were still active. Elsewhere in Thailand (Cholburi Province) the observed peaks in biting activity by balabacensis have occurred between midnight and 0300 hours when human activity has ceased. Finally, the question of the identity of the malarial parasites found infecting the balabacensis at Sadao poses a problem, for it cannot be determined on morphological grounds whether the parasites are human or simian. Anopheles balabacensis is known to serve as the natural vector of several simian malarias, and the forests surrounding the village in Sadao abounded with both monkeys and gibbons. The possibility exists also that some of the villagers there may have had infections of simian malaria.

Experimental infection of Anophelines with human malaria Since these studies were begun a total of 40 cases of falciparum malaria and 16 of vivax malaria have been exposed to laboratory reared Anopheles balabacensis and/or A. stephensi. A. balabacensis and A. stephensi were equally susceptible to both species of malaria. On the average, falciparum cases were more infective than vivax cases. The proportion of mosquitoes infected with falciparum gametocytes rose rapidly with increasing numbers of gametocytes ingested, reaching a peak at about 3000 gametocytes per cubic millimeter; thereafter it declined somewhat and stabilized at gametocyte levels of about 5000-6000 per cubic millimeter (Fig. 1.)

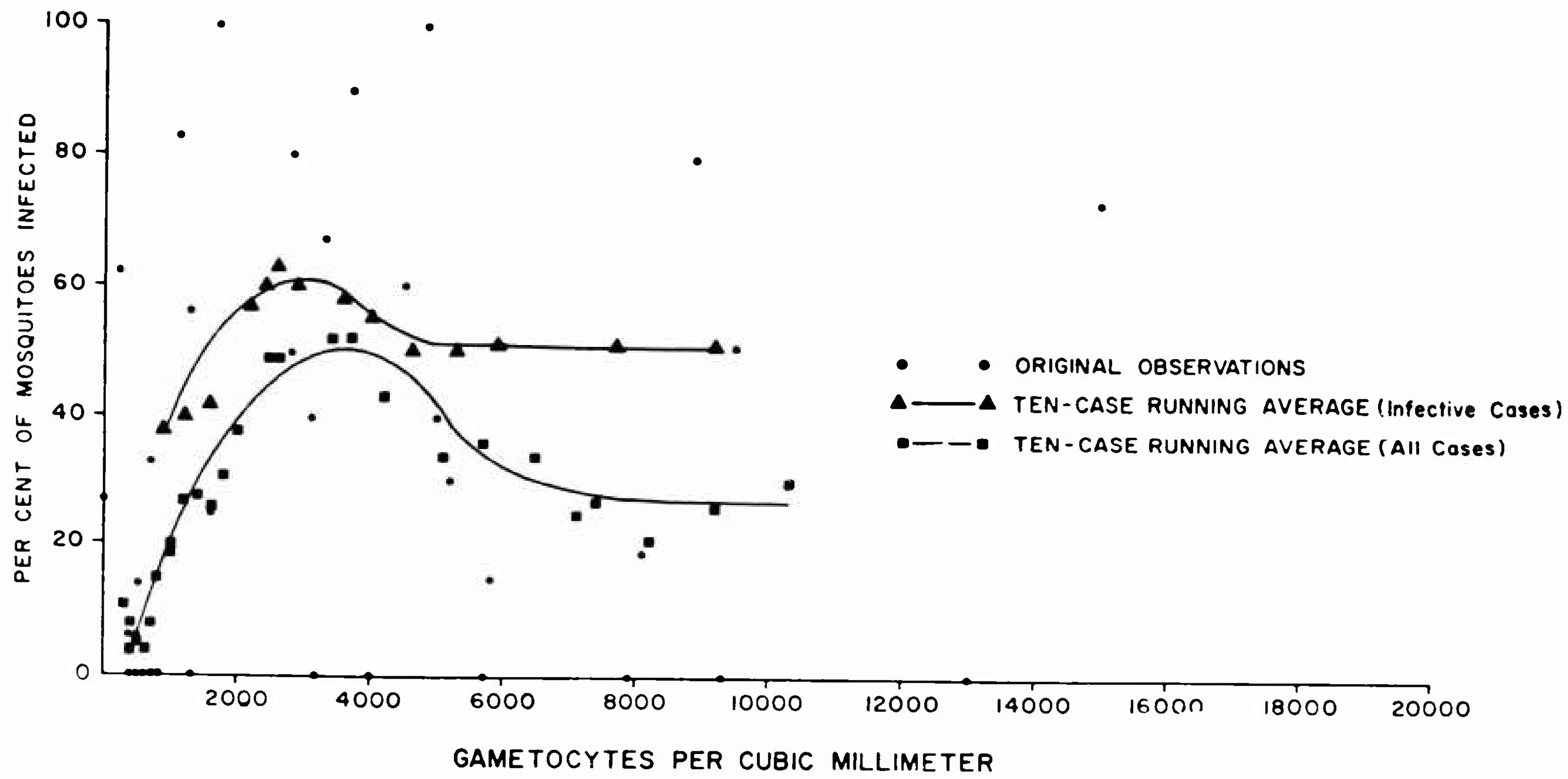
Table 3. Anopheles mosquitoes collected biting man outdoors between 1800-2400 at Sadao District 6-20 June 1966 (13 nights)

Anopheles aconitus	1
Anopheles balabacensis	214
Anopheles barbirostris	50
Anopheles indiensis	5
Anopheles karwari	10
Anopheles kochi	52
Anopheles maculatus	19
Anopheles philippinensis	129
Anopheles tessellatus	51
Total	531

Table 4. Pattern of human biting activity of A. balabacensis at Sadao District (13 Nights)-June, 1966

Hour	No. Mosquitoes Collected	Per Cent Total
1800-1900	8	4%
1900-2000	121	56%
2000-2100	40	19%
2100-2200	20	9%
2200-2300	21	10%
2300-2400	4	2%

Fig. 1. Proportion of mosquitoes infected following engorgement on falciparum malaria carriers.



Falciparum cases with high densities of asexual parasites tended to infect higher proportions of mosquitoes (Table 5). The mechanism of this effect is unknown, since asexual parasitemia was not correlated with gametocytemia. Insufficient data was obtained from vivax cases to allow for a similar comparison. A tendency of the older falciparum carriers to infect higher proportions of mosquitoes than the younger carriers was observed, but this was not due to higher gametocytemias since gametocytemia was not correlated with age. (Table 6). In falciparum malaria where was a distinct trend for cool season cases to be more infective for mosquitoes than hot or rainy season cases (Table 7). It appears that this can be explained on the basis of carries gametocytemia levels, for in nine out of ten falciparum cases encountered in the cool season gametocyte levels were higher than the median gametocytemia (2250/mm^3) for falciparum cases. The analogous tabulation for vivax cases was inconclusive; however, all four vivax cases encountered in the cool season had gametocytemias higher than the median level (700/mm^3) for vivax cases.

Table 5. Relation of asexual parasitemia to infection of mosquitoes in falciparum malaria.

Asexual Parasites per cmm	Cases		Mosquitoes					
			Infective Cases			All Cases		
	Infective	Total	Diss.	Pos.	%Pos.	Diss.	Pos.	%Pos.
< 1000	10	15	122	44	36	222	44	20
> 1000	7	14	90	62	69	192	62	32

Table 6. Relation of carrier age to infection at mosquitoes in falciparum cases.

Carrier Age	Cases		Mosquitoes					
			Infective Cases			All Cases		
	Infective	All	Diss.	Pos.	%Pos.	Diss.	Pos.	%Pos
< 20	12	21	164	62	38	256	62	24
> 20	12	19	130	74	57	263	74	28

Table. 7 Relation of season to infection of mosquitoes in falciparum malaria.

Season	Cases		Mosquitoes					
			Infective Cases			All Cases		
	Infective	All	Diss.	Pos.	%Pos.	Diss.	Pos.	%Pos.
Hot (Feb..May)	3	8	42	20	48	101	20	20
Rainy (Jun..Oct)	13	22	139	58	42	265	58	22
Cool Nov.. Jan)	8	10	113	58	51	153	58	38

Experimental malaria in lower primates During this period 42 attempts were made to infect mosquitoes by allowing them to feed on splenectomized gibbons with falciparum gametocytemias. Gametocyte concentrations in these gibbons ranged from less than 50 to 160,000 per cubic mm. A total of 23 individual gibbons have been used; five of them were used two or more times, at different stages of their individual infections, including consecutive daily feedings on three animals and seven feedings covering a 24-hour period at four hour intervals on one of them. Six strains of P. falciparum were involved, and these had been passed in gibbons from 1 to 20 times. Special procedures tried included post-feed incubation of mosquitoes at low and high temperatures, prefeeding of mosquitoes on normal blood and in vitro feeding of infected gibbon blood diluted with normal blood or with the plasma replaced with normal plasma. Both A. balabacensis and A. stephensi from laboratory strains were fed, and each of these species were known to be fully susceptible to falciparum malaria through concurrent experiments with human cases. All the above attempts have been unsuccessful.

As the result of the failure of attempts to infect mosquitoes on gibbons infected with falciparum malaria, experiments were undertaken to determine the stage at which the parasite fails to complete its development following ingestion by the mosquito. The exflagellation of microgametocytes was demonstrated in four of six falciparum-infected gibbons, but none of these animals were infective for mosquitoes fed upon them. Exflagellation was also observed, incidentally, in an ordinary thin smear prepared at the time of another feeding. Three series of attempts to find ookinetes in the guts of mosquitoes fed on gametocytemic gibbons, made at two hour intervals up to the 24th following blood meal ingestion, were negative. In the case of a single human infection, ookinetes were found in the mosquito gut within the first two hours of searching. Deformed gametocytes were more common in gut contents of mosquitoes fed on gibbons, and gametocytes became very difficult to find within 24 hours following ingestion.

A third gibbon (S-27) inoculated with falciparum sporozoites became patently positive after an incubation period of approximately 70 days. Two earlier successful attempts became patent after incubation periods of 44 and 45 days, respectively. Parasite levels in gibbon S-27 were highest on day 126 following inoculation, reaching 1500 parasites per 500 leucocytes. No parasites have been seen in this animal since day 147.

Summary Entomological and malaria studies conducted in the vicinity of vivax malaria foci in Phatumthani Province in the central plain of Thailand indicated that Anopheles aconitus is a vector in that region. That species, as well as A. philippinensis, A. tesselatus and A. vagus exhibited pronounced exophilic biting behavior; on the other hand, A. campestris was frequently collected biting indoors in that area. Studies in Sadao district, Songkla Province, near the Thai-Malaysian border implicated A. balabacensis as the principal vector of malaria there. Biting activity of balabacensis was greatest between 1900 and 2000 hours in the Sadao district, whereas elsewhere in Thailand this mosquito exhibits much later (2400-0300) peaks in biting activity.

Anopheles balabacensis and A. stephensi were fed on falciparum and vivax gametocyte carriers in central Thailand. In falciparum cases the highest rate of infection of mosquitoes occured when the gametocyte density was 3000 per cubic millimeter. Falciparum carriers were more infective than vivax carriers. Cases tended to be more infective during the cool season, or if they high asexual parasitemias or were older than the median age.

Feedings of anophelines on splenectomized gibbons with blood-induced falciparum gametocytemias gave uniformly negative results. Gametocytes in gibbon infections are apparently immature and short-lived. The microgametocytes are capable of exflagellation, but the ookinete stage is not achieved.

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SEATO MEDICAL RESEARCH STUDY ON MELIOIDOSIS

Coordinator: Richard A. Finkelstein, Ph.D., Deputy Chief, Department of Bacteriology & Mycology

Principal Investigator: Richard A. Finkelstein, Ph. D.

Associate Investigators:
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4. Jon Goodan, SP4

Period of Report: Annual, 1 April 1966 31 March 1967

Objective: This study is designed to determine the presence and distribution of *Pseudomonas pseudomallei* in Thailand and to evaluate its importance as the causative agent of the disease, melioidosis.

Description: Our previous efforts (see last Annual Report) have provided information regarding the general distribution of *Ps. pseudomallei* in soil and water samples obtained in various areas of Thailand and on the association of serological reactivity of Thai people with the presence of the organism in the external environment. Efforts to recognize active cases of infection in Thailand have been entirely unsuccessful, although isolated cases have previously been reported in the Thai medical literature. The conclusion which appears almost unavoidable is that *Ps. pseudomallei* is, in nature, a saprophytic organism which only rarely causes disease and then perhaps because of unusual routes of infection, abnormally low host resistance, or a combination of these factors. Nevertheless, when melioidosis does occur, it is a grave matter to the patient. Therefore sc [illegible] fort has been directed to examining experimental infection and the possibility of developing a more effe[illegible] therapeutic regimen. Because of the daparture of supporting personnel and the fact that little further can be gained by continuing the geographic and serological surveys, these activities have been deemphasized although the capability for isolation of *Ps. pseudomallei* and diagnosis, both bacteriological and serological, is being maintained. The laboratory still serves as a consultation laboratory for confirmation of isolates in Viet Nam and other areas and receives serum for serological study by the micro-hemagglutination test developed here. An extensive comparison of this serological test with that in use at WRAIR is in progress. The Thai Livestock Department has assumed some responsibility for further study of melioidosis, particularly in its veterinary aspects, using methodology and personnel developed and trained in this laboratory.

1 On loan from Thai Department of Health
2 Research and Education Division, Livestock Department, Ministry of Agriculture.

Progress:

1. Specimens received.

An additional 75 sputum samples, from supposedly non-tuberculous chronic and subacute pulmonary disease patients in Southern Provinces of Thailand (Pattalung, Songkhla, Pattani, Yala and Narathivas), where isolation rates from soil and water samples range from 25 to over 40%, have been examined for *Ps. pseudomallei* by hamster inoculation and cultural techniques. All were negative.

Thirty five specimens obtained from 31 swine at slaughter houses in the same region, and one cattle specimen, were also examined with negative results.

Nine natural water samples from Chandhaburi Province were negative for *Ps. pseudomallei.*

Eighteen cultures, representing 12 cases among U.S. servicemen in Viet Nam and one strain reportedly isolated from a rat, were confirmed as *Ps. pseudomallei.*

Results of antibiotic sensitivity tests of the isolates from Viet Nam, performed by the disc technique, were generally quite similar to those obtained with isolates from Thailand.

Sensitive	Resistant
Chloramphenicol, 30 μg	Penicillin, 5 U
Neomycin, 30 μg	Polymyxin B, 300 U.
Tetracycline, 30 μg	Furadantin, 15 μg
Kantrex, 30 μg	Erythromycin, 5 μg
Novobiocin, 10 μg	Bacitracin, 10 U.
Ampicillin, 15 μg	Methicillin, 5 μg
Sulfathiazole, 1.0 mg	Colimycin, 5 μg
	Streptomycin, 5 μg

One strain was found to be resistant to chloramphenicol and one to neomycin.

Virulence tests were performed by inoculating hamsters intraperitoneally with increasing dilutions of suspensions of three of the strains. The results indicated that the strains were highly virulent for hamsters with LD_{50} values between 1 and 10 viable organisms.

Two hundred twenty-one sera from healthy American servicemen in Viet Nam were tested for melioidosis antibody by the sensitized erythrocyte micro-hemagglutination technique. None were reactive.

Sera from six confirmed cases were also tested. Of these, 4 showed either a rise in titer in paired specimens or a significant titer in a single (late) specimen.

Sera from 9 Special Forces soldiers in Viet Nam, who had been for prolonged periods in an area which yielded cases, were negative in the HA test.

2. Attempt to enhance susceptibility of a naturally resistant host.

In a discussion of the melioidosis problem with COL W.D. Tigertt, COL S. Vivona and LTC R.L. Taylor, the question arose as to whether some factor may be increasing susceptibility of servicemen in Viet Nam to melioidosis. A common denominator is the use of chloroquine. Accordingly, an attempt was

made to determine the effect of chloroquine on the resistance to melioidosis of a naturally resistant species, the rat. Initially, LD_{50} assays were conducted in rats to determine the maximum sub-lethal dose of chloroquine and to ascertain their susceptibility to melioidosis. In these tests, it was found: 1) that rats would tolerate 2 subcutaneous doses at a 3 day interval of 1.0 ml of a 0.5% solution of choloquine (Resochin-Bayer), higher doses resulted in some deaths, usually almost immediately; and 2) that the LD_{50} for rats inoculated intraperitoneally was approximately 10[illegible] organisms, the rats dying by 40 hours.

In the experiment to determine the effect of chloroquine, the rats were divided into 2 groups, one group receiving 0.5 ml of a 0.5% solution of chloroquine subcutaneously on day 0 and day 2, and both groups receiving graded doses of Ps. pseudomallei intraperitoneally. The results are summarized in Table. I. Chloroquine apparently had a slight effect on enhancing susceptibility to the early (toxic?) deaths but no effect on susceptibility to delayed (infection type) deaths.

3. Attempt to enhance with chlorpromazine the effect of chloramphenicol.

In the same discussion (Par. 2, above), mention was made of the clinical failure of antibiotics which are effective *in vitro*. Col. Tigertt suggested that some attempt might be made to enhance the activity of antibiotics and recalled a report of an adjuvant effect of chlorpromazine on antibiotic action against *Brucella*. Accordingly, an attempt was made to determine whether chlorpromazine would enhance the effect of chloramphenicol in melioidosis infected hamsters.

The experiment was performed in 3 experimental groups of 20 hamsters, which were subdivided into sub-groups of 5 hamsters each, and a control group of 10 hamsters. The experimental and control groups each received an intraperitoneal inoculum of approximately 1×10^7 viable *Ps. pseudomallei*. On each of the next 3 days, the experimental groups received 2 doses of either chloramphenicol, chlorpromazine, or both. Appropriate control groups were inoculated with the drugs alone to observe any toxicity. Mortality was recorded twice daily. There were no deaths in the drug control groups. The results, summarized in Table II, did not indicate that chlorpromazine had any significant enhancing effect on chloramphenicol in this model although there was one long term survivor in the group treated with both drugs.

4. Effect of antiserum, alone and in combination with chloramphenicol, on experimental melioidosis in hamsters.

The disappointing results of the previous experiment, and especially the lack of absolute therapeutic effect of chloramphenicol, the drug of choice for this disease and one to which the infecting strain was sensitive *in vitro*, led us to test the effect of hyperimmune serum, alone and in combination with chloramphenicol, on the experimental infection of hamsters. The serum used was a pool of rabbit sera prepared against formalinized antigens of a variety of strains of *Ps. pseudomallei* and which had an HA titer of 1.20, 480. In this experiment, the dosage of chloramphenicol was increased 4-fold and therapy was initiated earlier following challenge. The results (Table III) were similarly disappointing. In the dosage used, the serum had little, if any, effect on the outcome of the experimental infection, and did not enhance the slight therapeutic effect (manifest as a delay in time of death) of chloramphenicol.

Summary:

1. We have, as yet, been unable to find active cases of melioidosis in Thailand despite the widespread presence of the causative organism in soil and water and the presence of antibody in people in endemic areas. Isolates of *Pseudomonas pseudomallei* from American servicemen in Viet Nam have been confirmed in this laboratory. They appear similar, in every way tested, to isolates from soil and water in

Thailand. Four of six proven cases demonstrated either a rise in antibody or a high titer in a single available specimen in the microhemagglutination test for melioidosis antibody developed here. In contrast, the sera of normal American servicemen in Viet Nam had no activity.

2. Chloroquine was found to have little effect on the natural resistance of the rat to melioidosis.

3. Chlorpromazine did not enhance the slight therapeutic effect of chloramphenicol on experimental melioidosis in hamsters, nor did hyper-immune pooled anti-*Pseudomonas pseudomallei* rabbit serum.

Table I

Effect of Chloroquine on Susceptibility of Rats to Melioidosis

Experimental Group	Inoculum*	Days following infection				
		1	2	7	9	17
Ps. pseudomallei only	2.6×10^8	6/8**				6/8
	2.6×10^7	2/8		2/8	3/8	3/8
	2.6×10^6	3/8				3/8
Ps. pseudomallei +	2.6×10^8	8/9				8/9
Chloroquine***	2.6×10^7	6/9	7/9			7/9
	2.6×10^6	1/9				1/9
	2.6×10^5	1/8				1/8
Chloroquine only***		0/8				0/8

* Viable cells in 0.1 ml intraperitoneally

** No. dead/Total

*** 0/5 ml of 0.5 % solution, subcutaneously, on initial day and 2 days later.

Table II

Effect of Chlorpromazine on Activity of Chloramphenicol in Infected Hamsters

Experimental Group*	Cage	Days following infection 1	2	3	4	11
Ps. pseudomallei, only	1	1/5**	5/5			
	2	0/5	5/5			
	Cumulative % Mortality	10	100			
Ps. pseudomallei +	1	0/5	3/5	5/5		
Chloramphenicol***	2	0/5	4/5	5/5		
	3	0/5	5/5			
	4	0/5	2/5	5/5		
	Cumulative % Mortality	0	70	100		
Ps. pseudomallei +	1	0/5	5/5			
Chlorpromazine****	2	0/5	5/5			
	3	0/5	5/5			
	4	0/5	5/5			
	Cumulative % Mortality	0	100			
Ps. pseudomallei +						
both drugs*****	1	0/5	3/5	4/5	5/5	
	2	0/5	2/5	4/5	4/5	4/5
	3	0/5	5/5			
	4	0/5	4/5	5/5		
	Cumulative % Mortality	0	70	90	95	95

* Each group received 1 x 12[illegible] viable Pseudomomas pseudomallei intraperitoneally on day 0.
** No. dead/Total
*** 5.0 mg, intramuscularly, at 0800 hours and 1600 hours on day 1,2, and 3.
**** 0.1 mg as above.
***** Mixture of both drugs, as above.

Table III

Effect of Pooled Hyperimmune *Ps. pseudomallei* Antiserum and Chloramphenicol, Alone and in Combination, in Infected Hamsters

Experimental Group[1]	Cage	Days following Infection 2	3	4	6
Ps. pseudomallei only	1	5/5[2]			
	2	5/5			
	3	5/5			
	4	4/5	5/5		
Cumulative % Mortality		95	100		
Ps. pseudomallei + serum[3]	1	5/5			
	2	5/5			
	3	5/5			
	4	4/5	4/5	4/5	5/5
Cumulative % Mortality		95	95	95	100
Ps. pseudomallei + Chloram.[4]	1	0/5	1/5	5/5	
	2	0/5	3/5	5/5	
	3	0/5	3/5	5/5	
	4	0/5	4/5	4/6	5/5
Cumulative % Mortality		0	55	95	100
Ps. pseudomallei + serum +	1	0/5	4/5	5/5	
chloramphenicol	2	0/5	3/5	4/5	5/5
	3	0/5	4/5	4/5	5/5
	4	0/5	2/5	3/5	5/5
Cumulative % Mortality		0	65	80	100

1 Each group received 9.5×10^1 viable *Ps. pseudomallei*, intraperitoneally on the morning of day 0.
2 No. dead/Total
3 0.2 ml per hamster, subcutaneously, 6 hours after challenge.
4 20 mg/hamster, intramuscular, 6 hours after challenge and twice daily thereafter.

SEATO MEDICAL RESEARCH STUDY ON NON-MARINE AQUATIC MOLLUSCA

Coordinator:	Rolf A.M. Brandt
Principal Investigator:	Rolf A.M. Brandt
Associate Investigators:	Brasong Temcharoen Suchat pariyanond

Period of Report: 1 April 1966–31 March 1967

GENERAL INFORMATION

The principal Investigator is associated with the SEATO Medical Research Laboratory through an Army Medical R & D Grant give to the University of Medical Sciences in Bangkok to provide for his services for a second period of three years. It was understood, that he should not only work as a Medical Malacologist and Parasitologist for the SMRL but also a teacher of Thai students at the local University.

During the three years of the first grant a careful survey of the non-marine aquatic molluscan fauna of Thailand was carried out in all 71 provinces of the kingdom and a considerably large reference collection was built up. This collection will be stored partly id the building of the Thai Research Council and partly in the U.S. National Museum.

Three Thai students were attached to the Department of Medcial Zoology for training in Medical Malacology and Parasitology. They have here successfully worked on their theses for obtaining the degree of Master of Science and two of them are now working as associate investigators in medical malacology and parasitology.

Although the second grant is primarily supposed to help research on larval stages of trematodes, studies on molluscs will be continued. As the general faunistic survey of the kingdom was concluded in March 1967 these studied will cover anatomy, variability, biology and ecology. Further collecting of molluscs will have the predominant objective of obtaining larval stages of trematodes for their further study. These studies of cercariae is carried out in cooperation with the School of Tropical Medicine in Bangkok.

Title: Non-Marine Aquatic Molluscan Fauna of Thailand

Principal Investigator: Rolf A.M. Brandt

Associate Investigators: Brasong Temcharoen
Suchat Pariyanond

Objective: As even a cursory study of helminthic diseases has been proved to be impossible without an intensive knowledge of the intermediate hosts of the worm parasites, an Army Medical R&D Grant was given to the University of Medical Sciences in Bangkok to provide for the services of a Medical Malacologist and Parasitologist for a period of three years. This grant had been extended for a second period of three years.

The objective of this grant was to make a careful survey of the non-marine aquatic molluscan fauna of the Kingdom, to build up a reference collection of the found species, to train Thai students in medical malacology and to help the SMRL and agencies of the Universities with the identification of collected materials. The reference collection, partly stored in Bangkok, partly in Washington, comprises now about 2000 lots, including many undescribed species.

Description: The survey of Thailand for Fresh-water Molluscs was continued in the last twelve months and about 50 new species–partly undescribed–were found. A more careful study of the brackish water fauna resulted in the detection of a surprisingly large number of species new for the Kingdom. Few of these species, however, are of importance for parasitology. In Egypt, a brackish water species of the prosobranchiate family of Potamididae (Pirinella conica) is known to harbour the cercariae of Heterophyes. Large numbers of Cerithidea, the closest relative of the above species, were collected and placed in dishes. No heterophoid cercariae were shed. As Echinostomatidae were reported from fishing villages in Thailand, and as the fresh-water molluscs, which are known to harbour metacercariae of Echinostomatidae, are rarely eaten by the villagers, extensive study of the brackish water molluscs which they do eat was carried out. No infected specimen was found. A detailed account of the species is given below.

With regard to the study of potential intermediate hosts of Schistosoma, particular care was focused on the genera Tricula, Ferrissia and Pachydrobia. Tricula had been reported as intermediate host of Paragonimus, but this report proved to be erroneous, as DAVIS proved that these so-called Tricula were small forms of Oncomelania. As it was evident that Tricula and Oncomelania look morphologically so similar that experienced taxonomists confound them, the local species of Tricula were carefully studied to point out the differenced from Oncomelania.

From India, reports are known of a Ferrissia species (fresh-water limpet) serving as intermediate host of Schistosoma. Therefore our local species of that genus were carefully studied, particularly as one of the few localities known of that genus coincides with a Schistosoma focus in Thailand. Although information was received from the Medical Department of the British Colonial Office that the above report was a "hoax", this study is being carried on because a fork-tailed cercaria was found in one of these species.

After several reports of cases of schistosomiasis from towns on the Mekong River were received, the fluviatile molluscan fauna of that river was closely studied, particularly those species of Pachydrobia and its relatives which were reported to us as "Oncomelania".

The principal investigator was invited by the Department of Zoology of the University of Malaya for a teaching visit and to help find the intermediate host of Opistorchis around Kuala Lumpur, as several cats were found infected with the liver-fluke. Although no infected snails were found, it can be assumed that Bithynia laevis, the only widely distributed Bithyniidae around Kuala Lumpur, serves as intermediate host, as the same species was found infected in Bangkok. The local food habits prevent infection of humans.

Progress and Reseults: These are shown by the following charts. As the collected specimens have been compared with material reported from other countries and some have been compared with material in different museums, a chart on zoogeography and ecology has been added. This gives the names of new species not included in the lists of the last report.

TABLE I

Geloina Species Examined for Metacercariae

Species	Amount	Locality	Date	Metacercariae
G. bengalensis LAM	71	Chantaburi	21–4–66	None
G. bengalensis LAM	19	Trad	26–7–66	"
G. bengalensis LAM	112	Takua Pa	29–8–66	"
G. bengalensis LAM	62	Grabi	22–8–66	"
G. bengalensis LAM	76	Satun	27–8–66	"
G. bengalensis LAM	37	Kao Yoi	14–6–66	"
G. impressa DESH	18	Grabi	22–8–66	"
G. impressa DESH	23	Satun	27–8–66	"
G. impressa DESH	22	Takua Pa	29–8–66	"
G. impressa DESH	16	Trad	26–7–66	"
G. impressa DESH	6	Grabi	23–8–66	"
G. impressa DESH	11	Tachalaeb	26–7–66	"
G. siamensis PRIME	48	Glaeng	21–12–66	"
G. siamensis PRIME	17	Kao Yoi	14–6–66	"
G. galathea MOERCH	69	Takua Pa	29–8–66	"
G. proxima DESHAYES	9	Trad	26–7–66	"
G. proxima DESHAYES	35	Glaeng	21–12–66	"
G. proxima DESHAYES	42	Takua Pa	29–8–66	"
G. proxima DESHAYES	8	Nara Tiwat	no date	"
G. proxima DESHAYES	12	Kantang	24–8–66	"
G. coaxans GMELIN	14	Chantaburi	21–4–66	"
G. coaxans GMELIN	7	Trad	27–7–66	"
G. coaxans GMELIN	52	Bandon	19–9–66	"
G. coaxans GMELIN	12	Satun	27–8–66	"
G. coaxans GMELIN	37	Takuapa	29–8–66	"
G. coaxans GMELIN	6	Trad	17–11–66	"
Six Species	843			

TABLE II

Cercariae and Metacercariae in Potamides and Telescopium

Species	Amount	Locality	Date	Species of Cercariae	Species of Metacercariae
P. eurypterrs ADAMS (?)	642	Welu River	29–7–66	1	0
P. palustris LAMARCK	766	Tachalaeb	26–7–66	0	0
T. telescopium LINNE	52	Khlung	29–7–66	0	0
T. telescopium LINNE	8	Narativat	4–4–66	0	0
T. telescopium LINNE	36	Grabi	21–8–66	0	0
T. telescopium LINNE	4	Trad	30–7–66	0	0
T. moritso BUTOT	12	Grabi	21–8–66	0	0
Potamides (Terebralia)	1408			1	0
Telescopium	110			0	0

TABLE III

Cercariae and Metacercariae in Cerithidea

Species	Amount	Locality	Date	Species of Cercariae	Species of Metacercariae
C. djadjariensis M.	120	Kao Yoi, Petburi	15 6–66	0	0
" "	942	Tachalaeb	21–4–66	0	0*
" "	1117	Klong Na Klua	10–1–67	0	0*
" "	433	Ban Ampoe	6–1–67	0	0
" "	1422	Klong Bang La Mung	4–1–67	1	0*
C. cingulata GMELIN	2361	Klong Na Klua	10–1–67	2	0*
" "	1860	Klong Bang La Mung	4–1–67	1	0*
" "	45	Tachalaeb	21–4–66	0	0
" "	648	Bang Saen	12–1–67	0	0
" "	104	Kao Yoi	15–6–66	0	0
" "	374	Klong Nachon Tian	26–4–66	0	0
" "	252	Grabi	23–8–66	0	0
" "	709	Ko Samui	20–9–66	0	0
" "	126	Trad	21–4–66	0	0
" "	20	Ban Ampoe	6–1–67	0	0
" "	1312	Sriracha	12–1–67	1	0*
" "	581	Glaeng	7–1–67	0	0*
C. alata PHILIPPI	291	Tachalaeb	21–4–66	0	0*
C. weyersi DAUTZ.	103	Kantang	25–8–66	0	0
C. charbonieri PETIT	35	Bandon	15–9–66	0	0
C. obtusa LAMARCK	10	Surat Thani	20–9–66	0	0
" "	80	Penang	no date	0	0
" "	50	Bandon	21–9–66	0	0
" "	6	Ban Tamru	21–1–67	0	0
" "	652	Satun	4–1–66	0	0*
" "	125	Pak Panang	23–11–65	0	0
" "	25	Tachalaeb	21–4–66	0	0
" "	277	Ranong	20–8–66	0	0
" "	163	Grabi	22–8–66	0	0
" "	413	Ko Samui	22–9–66	0	0
C. quadrata SOWERBY	481	Tachalaeb	21–4–66	0	0*
" "	2278	Ban Ampoe	6–1–67	1	0*
" "	88	Ban Tam Niap	7–1–67	0	0
" "	291	Ban Na Klua	3–1–67	0	0*
Seven species	16994				0
C. djadjariensis	4034	(1 specimen infected 0.025%)			0
C. cingulata	8392	(4 specimen infected 0.05%)			0
C. obtusa	1401	(0 specimen infected 0%)			0
C. quadrata	2958	(1 specimen infected 0.033%)			0

0* only 20% of the specimens were crushed

Table IV

Non-Marine Molluscan Fauna of Thailand Brackish and Fresh-water Species
Zoogeography and Ecology

Species		Thail. and				Asia										Fresh Water				Br. W		Remarks
	Thailand only	Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo-Pacific	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks	fluviatile	lacustrine	still water	estuarine	mud flats	
<u>Neritidae</u>															X	X	X	X		X	X	predominantly marine
<u>Neritina</u>												X				X	X	X		X	X	
pulligera L.								X	X							X	X					
chimmoi REEVE						X	X		X	X											X	sulcata LAM.?
variata LESS						X	X	X	X												X	
violacea LAM.						X	X	X	X	X											X	
<u>Clithon</u>												X								X	X	rarely marine
sowerbyana R.						X	X	X	X	X										X		and in lagoons
oualaniensis						X	X	X	X											X		
<u>Nerita</u>												X								X	X	predominantly marine
lineata SOW.						X	X	X	X	X										X	X	
planispira PFR						X	X		X												X	
<u>Viviparidae</u>															X	X	X	X	X			not South America
<u>Idiopoma</u>						X	X									X	X	X	X			
umbilicata LEA							X										X	X	X			
variata FRFLD						X	X									X	X	X				
<u>Cipangopoludina</u>						X	X	X								X	X	X	X			
crooki BR.	X			?													X					
<u>Anulotaia</u>				X	X												X					one other species in Cambodia
forcarti BR	X																X					
<u>Trochotaia</u>				X	X													X	X			
trochoides M.				X	X													X	X			
<u>Eyriesia</u>				X	X												X	X				monotypical genus
eyriesi DESH.				X	X												X					
<u>Sinotaia</u>						X	X	X	X	?												
guangdungensis							X	X									X					
<u>Siamopaludina</u>						X	X	X	X	X												
martensis FRF.							X	X	X								X	X	X			
javanica HASS.							X		X									X	X			
zilchi BR.				X													X					
maekokensis BR.		X		X													X		X			

Species	Thailand only	Thail. and Burma	Malaya	Laos	Cambodia	Asia South	Southeast	East	Insulinde	Australo–Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	Fresh Water creeks	fluviatile	lacustrine	still water	Br. W estuarine	mud flats	Remarks
Filopaludina HABE						X	X									X	X	X	X			& Madagaskar
filosa REEVE		?		X													X		X			
doliaris GOULD		X				X												X	X			
polygramma MART.			X		X												X	X	X			
sumatrensis							X		X													ssp. speciosa DESH. & South Vietnam
Mekongia				X	X												X					
siamensis FRFLD.	X																X					Mae Klong
bocourti MABILLE	X																X					Petburi river
bourguignati ,,	X																X					''
heinesiana LEA	X																X					
swainsoni LEA				X	X												X					
moreleti DESH.				X	X												X					Mekong
rattei FISCHER				X	X												X					Mekong
lamarcki DESH.				X	X												X					Mekong
pongensis BR.				X													X					
flavida BRANDT				X													X					
Ampullariidae												X					X	X	X			
Pila						X	X	X	X	X								X	X			
ampullacea L.						X	X	X	X	X								X	X			
polita DESH.						X	X	X	X										X			
callistoma MORELET							X											X	X			
scutata MOUSSON						X	X		X									X	X			
gracilis LEA				X	X													X	X			
Bithyniidae															X	X	X	X	X			
Gabbia											X							X	X			
wykoffi BRANDT	X	?	?	?	?													X	X			
pygmaea PRESTON		X				X										X						
cf. longicornis							X												X			
Bithynia															X		X	X	X			subgen. Digonio–stoma
funiculata WALKER		X		X															X			
goniomphalus MOR.							X	?									X	X	X			
siamensis LEA				X	X												X	X	X			
laevis LEA					X													X	X			& Malaya
pulchella BENSON						X											X	X	X			
Wattebledia							X		X									X	X			
crossean WATTEBL.							X											X	X			not Java
siamensis MLLDFF.	X																	X	X			

Species	Thailand only	Thail. and				Asia										Fresh Water				Br. W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo-Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks	fluviatile	lacustrine	still water	estuarine	mud flats	
Bithyniidae cont.																						
Hydrobioides						X	X											X	X			/India, Burma,
dautzenberi WALK.				X																		Laos, Thl. /
Hydrobiidae															X	X	X	X	X	X	X	and marine
Pachydrodia							X										X					
siamensis BRANDT	X																X					end. Mae Klong
zilchi BRANDT	X																X					end. Mun River
munenses BRANDT																	X					end. Mun River
crooki BRANDT				X	X												X					Mekong
spinosa POIRIER				X	X												X					Mekong
tuberculata BR.				X													X					Mekong
harmandi POIRIER				X	X												X					Mekong
variabilis POIR.				X	X												X					Mekong
paradoxa CR. & F.				X	X												X					Mekong
Pachydrobiella				X	X												X					
brevis BAVAY				X	X												X					Mekong
dubiosa BRANDT	X																X					Mae Klong
Hubendickia				X	?												X					
siamensis BRANDT	X																X					end. Mun River
bandoni BRANDT				X													X					Mekong
Paraprososthenia							X										X					
schlickumi BR.	xx																X					end. Mun river
crooki BRANDT				X													X					Mekong
schuetti BRANDT				X													X					Mekong
Hydrorissoia				X	X												X					
elegans BAVAY				X	X												X					
Lacunopsis							X										X					
monodonta DESH.				X	X												X					ssp. munensis BR
harmandi POIRIER				X	X												X					
coronata HEUDE							X										X					dead shells only
radomani BRANDT				X													X					end. Mekong
Jullienia																						
tricostata DESH.				X	X												X					end. subspecies
crooki BRANDT				X													X					Mekong
Tricula						X	X	X								X	X					
bollingeri DAVIS	X	?														X						
burchi DAVIS	X	?														X						
Rehderiella	X																X			X		
parva LEA	X																X			X		
siamensis BRANDT	X																X			X		

Species	Thailand only	Thail. and Burma	Malaya	Laos	Cambodia	Asia South	Southeast	East	Insulinde	Australo-Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	Eresh Water creeks	fluviatile	lacustrine	still water	Br. W estuarine	mud flats	Remarks
Clenchiella						X	X		X	X										X	X	and tidal area
microscopica NEV.						X	X														X	and tidal area
Tornidae															X		X					predominantly marine
Chamlongia MS	X																				X	and tidal area
harinasutai BR	X																				X	and tidal area
Truncatellidae																				X		Sea coast, amphibious
Truncatella																				X		,, ,, ,,
guerini VILIA						X	X	X	X	X												,, ,, ,,
valida PFEIFFER						X	X	X	X	X												,, ,, ,,
Stenothyridae						X	X	X	X	X							X			X	X	
Stenothyra						X	X	X	X	X							X			X	X	
brasongi BRANDT	X																			X	X	
prashadi BRANDT	X																				X	
annandalei BR.	X																				X	
krungtepi BR.	X																			X		
mandahlbarthi BR.	X																			X		
glabrata ADAMS							X													X	X	
ventricosa Q. & G.							X													X	X	
schlickumi BR.	X																					
koruti BRANDT				X	?												X					
fasciata BRANDT	X																X					
crooki BRANDT	X																X					
wykoffi BRANDT	X																X					
roseni BRANDT	X																X					
schuetti BRANDT	X																X					
jlraponi BRANDT	X																X					
spiralis BRANDT	X																X					
Gangetica						X	X													X	X	
tigertti	X																			X	X	
Iravadiidae						X	X	X												X	X	
Iravadia						X	X	X												X	X	
siamensis BR.	X																					
ornata BLANFORD	X																			X	X	

Species	Thailand only	Thail. and				Asia										Fresh Water				Br. W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo - Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks	fluviatile	lacustrine	still water	estuarine	mud flats	
Fairbankiidae																						
Fairbankia						X	X	X	X											X	X	
berryi BRANDT			X																	X	X	
rohdei BRANDT			X																	X	X	
truncata BRANDT	X																			X	X	
Assimineidae															X					X	X	land and marine
Assiminea															X					X	X	mostly amphibious
brevicula PFFIFFER						X	X	X	X											X	X	
borneensis IoSEL							X		X											X	X	
nitida PEASE							X	X	X	X										X	X	
woodmasoniana NEV.						X	X		X											X	X	
javana THIELS							X		X											X	X	
microsculpta NEV.						X	X	X	X											X	X	
bandoni BRANDT	X																			X	X	philippinica subsp.?
semilirata BTTG.							X		X											X	X	
thonburi BRANDT	X																			X		and tidal area
daengsvangi BR.	X																			X		and tidal area
siamensis BRANDT	X																			X		
Paludinella													X							X	X	amphibious
carinata LEA	X				?															X		tidal area
fasciolata MOREL.	X																			X		
brunnea BRANDT	X																			X		
Thiaridae															X	X	X	X	X	X		except cold zones
Brotia H. ADAMS						X	X	X	X							X	X					
siamensis BROT		X	X			X			X								X					costata subspec.
citrina BROT	X	?		?												X	X					upper reaches
peninsularis BRAND			X													X	X					
pagodula GOULD		X															X					Moei River
binodosa BLANFORD	X																X					Maek Noi river only
armata BRANDT	X																X					
subgloriosa BR.	X																X					subsp. of binodosa
pongensis BRANDT	X																X					end. Maenam Pong
baccata GOULD		X														X	X					
pseudasperata BR.	X						?									X	X					Annam? Malaya?
? housei LEA				X	X												X	X				
Melanoides													X			X	X	X	X	X		
tuberculata MLL.												X				X	X	X	X	X		
jugicosta BENSON		X				X											X		X			

Species	Thailand only	Thail. and Burma	Malaya	Laos	Cambodia	Asia South	Southeast	East	Insulinde	Australo - Pacific.	Paleotropic	Circumtropic	Holarctic	Cosmopolitan	Fresh Water creeks	fluviatile	lacustrine	still water	Br. W estuarine	mud flats	Remarks
Thiaridae cont.																					
Tarebia						X	X	X	X	X					X	X	X	X			
granifera LAM.						X	X	X	X	X					X	X	X	X			
bangpraensis BRANDT	X															X	X				
Sermyla						X	X	X	X	X									X	X	
riqueti GRATELOU						X	X	X	X	X									X	X	
krungtepi BRANDT	X																		X		Maenam Chao Phaya
Thiara						X	X	X	X	X	?				X	X	X	X			East Africa
scabra O.F. MULLER						X	X	X	X	X					X	X	X	X			
Faunus						X	X	X	X	X						X			X		
ater L.						X	X	X	X	X						X			X		
Pleuroceridae														X	X	X	X	X			
Semisulcospira							?	X							X	X	X	X			
? housei LEA																X	X	X			
Paludomus						X	X	X	X	X					X						
siamensis BLANFORD	X														X						
petrosa GOULD		X													X						
burmanica NEVILL		X													X						
ornata BENSON		X													X						
Potamididae														X					X	X	except cold region
Cerithidea														X					X	X	
weyersi DAUTZENBERG						X	X		X										X	X	
churbonieri PETIT						X	X		X										X	X	
alata PHILIPPI							X		X										X	X	
obtusa LAM						X	X	X	X	X									X	X	
quadrata SOWERBY						X	X	X	X	X									X	X	
cingulata GMELIN						X	X	X	X	X									X	X	
djadjariensis MARTIN							X	X	X										X	X	
Telesc[illegible]						X	X		X	X									X	X	
telesc[illegible]						X	X		X	X									X	X	
morits[illegible]			X						X										X	X	telescopium frm.?
Terebralia						X	X	X	X	X									X	X	
palustris BRUG.						X	X	X	X	X									X	X	
spec. ind.							X		X										X	X	
sulcata BORN						X	X		X										X	X	
Margainellidae														X		X			X		marine
Rivomarginella BRANDT																					only fresh-water
morrisoni BRANDT	X															X					genus and species

Species	Thailand only	Thail. and Asia: Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo-Pacific	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	Fresh Water: creeks	fluviatile	lacustrine	still water	Br.W: estuarine	mud-flats	Remarks
Buccinidae															X							predom. marine
Clea							X	X	X								X					
cambodiensis SOW.					X		X										X					
siamensis. n.				X													X					
crooki n.				X													X					
Anentome							X									X	X	X	X			
baudoniana MAB. & LM							X									X	X	X	X			
Chicoreus						X	X	X	X	X											X	
capucinus LAM						X	X	X	X												X	
Allectrion						X	X	X	X											X	X	
taenuis GMELIN						X	X	X	X											X	X	
Littorinidae															X					X	X	marine
Littorinopsis						X	X	X	X	X										X	X	and marine
angulifera LAM.						X	X	X	X											X	X	
melanostoma GRAY						X	X	X	X											X	X	
Ellobiidae															X					X	X	and terrestral
Ellobium												X								X	X	
aurisjudae L.						X	X		X	X										X	X	
aurismidae L.						X	X		X	X										X	X	
Melampus												X								X	X	and amphibious
fasciatus DESH.						X	X	X	X	X										X	X	
singaporensis PFR.						X	X	X	X	X												coastal zone
siamensis PFR.	X																				X	
Laemodonta												X									X	
punctigera ADAMS						X	X	X	X	X											X	
siamensis MARTENS	X																				X	
Pythia											X										X	
trigonis TROSCHEL						X	X		X	X											X	
plicata BORN						X	X		X	X											x	
Cylindrotis							X		X												X	coastal zone
quadrasi MLLDFF.							X		X												X	
Cassidula						X	X	X	X	X											X	
aurisfelis BRUG						X	X	X	X	X											X	
mustelina DESH.						X	X		X												X	
Auriculastra						X	X	X	X	X											X	
subula QUOY & GAIM							X	X	X												X	

Species	Thailand only	Thail. and				Asia										Eresh Water				Br. W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East	Indonesia	Australo—Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks	fluviatile	lacustrine	still water	estuarine	mud.flats	
Atyidae															X					X	X	marine
Haminaea												X									X	marine
? perrieri MORLET							X														X	
Onchidiidae												X									X	
Onchidium												X									X	
verruculatum CUV.						X	X	X	X	X											X	
coriaceum SEMPER						X	X		X	X											X	
Lymnaeidae															X	X	X	X	X			
Radix															X	X	X	X	X			
rubiginosa MICH.							X		X							X	X	X	X			
luteola LAM						X	X										X	X	X			
viridis Q. & G.							X	X	X	X									X			
bowelli PRESTON						X	X	X								X						
Planorbidae															X	X	X	X	X	X		
Indoplanorbis						X	X		X	X						X	X	X	X			
exustus DESH.						X	X		X	X						X	X	X	X			
Gyraulus															X	X	X	X	X	X		
siamensis BRANDT	X																			X		
confusus ROCHEBR.						X	X	X	X	X						X		X	X			
convexiusculus H.						X	X	X	X	?						X	X	X	X			
cf. labiata BENSON						X	X									X						
spirillus GOULD						X	X									X			?			
Amerianna							X		X	X								X	X			
carinata H. ADAMS							X		X	X								X	X			Imported ?
Culmenella																						
jiraponi HUBENDICK	X																		X			
Helicorbis						X	X	X	X	X								X	X			
umbilicata BENSON						X	X	X	X	X								X	X			
Trochorbis						X	X	X	X									X	X			
trochoideus BENSON						X	X	X	X									X	X			
Polypylis						X	X	X	X									X	X			
hemisphaerula BENS						X	X	X	X									X	X			
Ancylidae															X	X	X	X	X			
Ferrissia										X	X					X	X	X	X			
javanica MARTENS							X		X									X	X			
krungtepi BRANDT	X																	X	X			
verruca BENSON						X										X						
baconi BOURGUIGNAT						X										X						

Species	Thailand only	Thail. and				Asia			Insuline	Australo-Pacific	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	Fresh Water				Br.W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East								creeks	fluviatile	lacustrine	still water	estuarine	mud-flats	
Pyramidellidae																						
Morrisonia MS	X														X					X	X	marine
krungtepi BRANDT	X																			X	X	
gracilis BRANDT	X																			X	X	
acicula BRANDT	X																			X		
bandoni BRANDT	X																			X	X	
spiralis BRANDT	X																			X		
Chrysallida																				X		
hanseni BRANDT	X														X						X	marine
Arcidae																					X	
Scaphula						X	X								X					X		marine
chaoprayaensis	X																			X		
Mytilidae																				X		=deltae BENSON
Modiolus															X		X	X		X		marine
siamensis MORLET				X											X		X	X		X		
evansi SMITH	X																X	X		X		
Anomiidae																		X				
Anomia												X								X	X	mangrove swamps
aenigmatica LAM						X	X		X	X		X								X	X	
Ostreidae																				X	X	
Alectryonia															X					X		marine
folium L.												X								X		marine
? gryphoides SCHLOTH.						X	X		X	X	X	?								X		
																				X	X	
Unionidae																						
Unio															X	X	X	X	X	X		
dorri WATTEBLED							X	X				X			X							
Oxynaia							X										X					
jourdyi MORLET							X										X					
																	X					
Quadrulidae																						
Pseudodon						X	X	X	X	X												
mouhoti LEA				X	X											X	X	X	X			
inoscularis GOUL						X	X									X	X					
vondenbuschi v. H.							X		X							X	X	X	X			
Trapezoideus						X	X									X	X		X			
folliaceus GOULD						X	X									X	X					
																X	X					

Species	Thailand only	Thail. and				Asia										Fresh Water				Br. W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo—Pacific.	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks	fluviatile	lacustrine	still water	estuarine	mud-flats	
Ensidens							X										X	X	X			
ingallsianus LEA							X										X	X	X			
Contradens							X		X							X	X	X	X			
ascia HANLEY			X													X	X					
semidecoratus MOREL				X	X												X	X	X			
tumidulus LEA				X	X											X	X					
rustica LEA					X													X				
Nannonaia						X	X	?	X								X					
pilatus LEA				X	X												X					
substriata LEA		?		X	X												X					
Pilbryoconcha						X	X		X								X	X	X	X		
lemeslei MORLET				X	X														X			
exilis LEA							X		X								X	X	X	X		
Unionetta																						
fabagina DESH. & J.				X	X												X					
Physunio						X	X		X								X					
eximius LEA				X	X												X					
gravidus LES			X						X								X					— superbus LEA?
Hyriopsis							X	X	X								X	X	X			
bialatus SIMPSON			X	X	X												X	X	X			
delaportei CR. & F.				X	X												X	X				
modelli BRANDT	X																X					
myersiana LEA			X	X	X												X	X				
Chamberlainia																						
hainesiana LEA	X			?	?												X					
Cristaria							X										X	X				
plicata LEACH							X										X	X				
Scabies						X	X										X	X	X			
scobinata LEA						X	X										X	X	X			
crispata GOULD							X										X					
phasellus LEA				X	X												X					
morrisoni BRANDT MS	X																X					
Pletholophus							X										X					
inangulatus HAAS				X	X												X					
Modellnaia	X																X					end. Mun River
munensis BRANDT	X																X					—
Solenaia						X	X	X											X			not yet found
emarginata LEA	X																		X			

Species	Thailand only	Thail. and				Asia										Fresh Water				Br.W		Remarks
		Burma	Malaya	Laos	Cambodia	South	Southeast	East	Insulinde	Australo Pacific	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	creeks & streams	fluviatile	lacustrine	still water	estuarine	mud.flats	
Corbiculidae												X					X	X	X	X		
Geloina						X	X	X	X	X										X	X	
bengalensis LAM.						X	X		X											X	X	
coaxans GMELIN						X	X		?											X	X	
siamensis PRIME		X			X															X	X	sumatrensis subsp.?
proxima PRIME		X																		X	X	
galathea MOERCH		X				X														X	X	Ceylon
impressa DESH.						X	X		X											X	X	
Batissa						X	X													X		
similis PRIME		X				X														X		
Corbicula											X					X	X	X	X	X		
aruta SOWERBY						X	X										X					
baudini MORLET							X										X					
blandiana PRIME				X	X												X					
bocourti MORLET							X										X	X	X			
castanea MORELET				X	X												X					
cyreniformis PRIME			X						X								X					
ducalis PRIME			X						X								X					
gubernatoria PRIME				X	X												X					
lamarckiana PRIME							X										X	X	X			
dautzenbergiana PR.				X													X					
moreletiana PRIME							X										X	X	X			
noetlingi MARTENS		X		X													X					
occidens BENSON							X										X					
ostiorum BRANDT	X																			X		
pisidiaeformis PR.	X																X					
regia CLESSIN			X														X					
salmonis BRANDT				X													X					
tenuis CLESTIN				X	X												X					
viridis BRANDT	X																					Chao Praya river
Sphaeriidae															X	X	X	X	X	X		
Pisidium															X	X	X	X	X	X		
javanum BENTHEM J.						X	X		X							X	X	X	X			
sumatrinum MARTENS			X						X							X						
clarkeanum NEVILL						X	X									X						
annandalei PRASHAD		X				X										X						
nevillianum THEOB.		X				X										X						

Species	Thailand only	Thail. and Burma	Malaya	Laos	Cambodia	Asia Insulinde	Australo-Pacific	South Asia	SE Asia	East Asia	Paleotropic	Circumtropic	Palearctic	Holarctic	Cosmopolitan	Eresh Water creeks & streams	fluviatile	lacustrine	still water	Br. W estuarine	mud-flats	Remarks
Psammobiidae															X					X	X	and marine
Psammotaea												X										— —
layardi REEVE								X	X											X	X	
Psammotella												X								X		and marine
spec. nov.?	X																			X	X	
Psammobia															X					X		and marine
violacea LAM.						X	X	X	X											X		
Glaucomyidae						X	X	X	X	?							X			X	X	
corrugata LAM								X	X											X		
krungtepi BR.	X																			X		
Solenidae															X		X			X	X	predominantly marine
Novaculina								X	X								X					
siamensis MORELET	X																X					
Pholadidae															X					X		predominantly marine
Martesia								X	X											X		
rivicula SOW	X																			X		
Teredinidae															X					X		predominantly marine
Nausitora																						
smithi BARTSCH	X																			X		

Summary and Conclusion.

Zoogeography. The general survey on non-marine molluscs in Thailand has been concluded in March 1967. In the four years of this faunistic survey 277 species of molluscs were found in fresh and brackish water, 97 in brackish or tidal water, 180 in fresh-water, five of which may also be found in estuarine water. Of these 277 species 75 species seem to be endemic for Thailand, at least they have not yet been found in other countries. It is understandable that this group contains the bulk of the undescribed species, 55 of 61. Eighty-three species are known from one or more neighboring countries, 118 are distributed over larger areas of Asia, Three of these species may be called paleotropic. No circumtropic or even cosmopolitan species is known from Thailand. All larger species and many of the smaller ones are related to parasitology, most of the local genera and almost all of the families have been proved to be of importance to human and veterinarian medicine. The description of the new species is in press, the complete fauna will be finished in about two months time.

Parasitology. Cases of Heterophyes, identified by a Japanese taxonomist as H. heterophyes, lead to the collecting of large numbers of Cerithidea, the closest local relative of the African intermediate host, Pirinella conica. Of nearly 17000 collected specimens, which belong to seven species, none has shed heterophoid cercariae. The cercariae which were found are not related to human or veterinarian medicine.

Echinostomiasis is widely distributed in Thailand, and also reported from coastal villages. As clams of the genus Geloina and snails of the genera Cerithidea, Terebralia and Telescopium are used as food in those areas several species of these genera were examined for metacercariae. In none of the 843 Geloinae and 1500 Potamididae did we find metacercariae. Therefore it is very improbale that these species serve as intermediate hosts. As among several thousand Cerithideae no infested snails was found, this genus can definitely be excluded from the list of potential second intermediate hosts. It seems highly improbable, that any brackish water species will be infested with metacercariae of Echinostomatidae, as the known first intermediate hosts are strictly confined to freshwater only.

The program for the next three months is the study of the cercariae shed by Ferrissia, Tricula and the Basommatophorae of the mud-flats, as some of these serve as food in Thailand.

SEATO MEDICAL RESEARCH STUDY ON MOSQUITOES

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Period of Report: 1 April 1966-31 March 1967

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STUDY REPORTS

1. Title	Mosquito Fauna of Thailand
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Objective To collect, identify, catalog and redescribe all of the mosquito species of Thailand. Information is also assembled on the distribution, larval habitats and other aspects of the bionomics of the various species. The eventual goal is the production of a monograph on the mosquitoes of the area, together with keys, handbooks and other identification aids for use of workers in public health and associated fields and the later inclusion of this material into a larger monographic work on the mosquitoes of Southeast Asia. Since the final monographs will not be completed for several years, periodic papers with keys, descriptions and distritution of important groups will be made available as soon as practical. The immediate objective is to make available as much information as possible on the Anopheles and species of other genera which are known or suspected to be vectors of disease. The training of competent Thai personnel and, more recently, U.S. military personnel in Southeast Asia in the identification and bionomics of the mosquito fauna of Thailand is also a major concern. Another objective is the building of a reference collection at SMRL of all the mosquitoes known to occur in Thailand to provide readily accessible study material to newly assigned and other personnel who may have the need for rapid familiarization with the mosquitoes in this area.

Description: Mosquitoes are collected from many areas of Thailand in connection with various studies on arthopod-borne viruses and malaria. Additional collections of a specialized nature are made to obtain correlated series of larvae, pupae and adults for illustration and other detailed studies. These consist mainly of collections of the immature stages from forested or undeveloped areas; these immature stages are reared individually, as far as is possible, in order to recover a correlated series cast skins and adults. All of the reared material is later identified and processed at SMRL in Bangkok. After processing, the material is transfered to the Southeast Asia Mosquito Project, U.S. National Museum for confirmation, description and eventual inclusion in the final monograph.

Progress: During the year 937 larval collections from 10 different provinces of Thailand were made. From these collections 7,115 adults were pinned and added to the collections. Each of these were reared individually and are represented by matched larval and pupal skins. Slide mounts were prepared from 10,993 larvae and larval and pupal skins. The transfer of material to the U.S. National Museum during the year included 95 boxes of pinned adults and 127 slide boxes of immature stages representing several hundred species and several thousand specimens.

Many Thai species not previously represented were added to the department collection this year. Several of these are new records for Thailand, while others require confirmation by examination of type material at the U.S. National Museum before a specific determination can be made. Much additional information on distribution and bionomics of approximately 160 species was also added. A breakdown of

this information for the Anopheles collected during the year is presented in Table 1. A project is underway to compile similar information on all species of mosquitoes collected by SMRL over the last few years and will be published as a separate report at a later date. Although the number of collectors in Table 1. are not suffficient to draw conclusions for all species there are a few points worthy of discussion. Of the 106 collections of mosquitoes made from elephant foot-prints, 41 contained larvae of Anopheles (Cellia) balabacensis, representing 66% of the 62 collections of balabacensis made during the year. Of the 5 species found in this type of habitat it was by far the most common. All of these foot-prints are found in forested areas in the hill or mountain regions, usually where elephants are used in logging operations but many, especially at the higher elevations are made by wild elephants. In Thailand the elephant foot-print is definitely a true habitat entry and is the specific habitat of several of the Culex (Culiciomyia) species. It can also be noted from the table that balabacensis strongly favors partially to heavily shaded habitats. It is usually not found in foot-prints that are completely exposed to sunlight or those that are not maintained in a very fresh state by rainfall or seepage. It could therefore be logically assumed that during rainy season those areas where the foot-prints are numerous and under forest cover become one of the primary sources of this species. It is also the only species collected at all levels of altitude from below 15 to above 1500 meters. An. (C.) minimus and An. (C) maculatus occured at all levels from below 150 to above 900 meters, both of these species are fresh water breeders, but show less preference for shade than balabacensis. Ground pools and flood pools in the broad sense are the same habitat. However flood pools are classified here as those small, usually shallow, temporary pools that are maintained in a very fresh state, primarily by frequent rainfall, and they frequently have one or more species of the genus Aedes present which are almost exclusively temporary flood pool breeders occuring only after heavy rains or flooding. Ground pools include both small to large, temporary or semipermanent pools with polluted, turbid or clear, but not necessarily fresh water. It can be seen that significant numbers of collections of maculatus and balabacensis were made only from flood pools; as the rains become less frequent neither of these species are found in this type of habitat. With the exception of An. (Anopheles) barbirostris and An. (C.) vagus, An. balabacensis and An. maculatus were found in a wider range of habitat than all other species. The greatest number of species were collected between the 15 to 150 meter level. All but five of these are commonly found in open agricultural or forest fringe areas and are commonly found breeding in rice fields or irrigation ditches. An. (An.) campestris occurs almost exclusively in the open agricultural plains, predominately in irrigation ditches. Two species are shown as habitat specific An. (An.) asiaticus and An. (A.) tigertti are known only from these type habitats. The localities listed for both species are new distribution records for Thailand. Previous to this report An. (A.) sintonoides was known to occur only in tree and stump holes. Many of the localities listed for the various Anopheles species are new distribution records for Thailand.

No one area of Thailand was surveyed for an extended period of time during the year and the number of collections from each area are insufficient to make a valid comparison on a regional basis. The areas selected for study, as shown in Figure I, represent to some extent a cross-section of the entire country. Species collected during the year that have not been listed in previous reports under this study are presented in Table 2. Some of these and others of special interest are briefly mentioned below.

Anophelines Anopheles (A.) asiaticus, discussed in above paragraph, was reported earlier in Thailand for the first time by SMRL from Tak province. Until this report period it was known only from that province. An. (C.) pampanai, was also earlier reported for the first time in Thailand by SMRL from Chanthaburi and Prachinburi province. The additional collection from the northern province of Nan is of special taxonomic interest. Determination of this species is important since it is so very closely related to An. (C.) minimus and some of the earlier records from these areas probably include pampanai. An. (A.) sintonoides the two collections of this species from Pandanus (screw pine) axils are of interest and somewhat of a surprise. As far as we can recall this is the first time an Anopheles has been collected from Pandanus axils. An. (A.)

tigertti, discussed briefly above, was first discovered by SMRL and previous to this report was known only from Prachinburi province. An. (C.) varuna: a very small number of adult specimens which appear to be this species still continue to be collected occasionally by SMRL. The species has been reported from Thailand but its correct identification and status has been questionable. The species is very close to several others of the minimus group, and the morphological characters used to separate members of this group exhibit a marked degree of variation within each species. We believe that the few adults being encountered and tentatively identified as varuna are in fact not varuna and are nothing more than variants of minimus. Two sibling series of minimus from Saraburi have been obtained and the adults from this series exhibit the full range of variables in wing characters used in identification. A small number of varuna (?) also occur in Saraburi and further attempts are to be made to recover eggs from some of these specimens. Recovery of a sibling series from one or more of these specimens should clear up the question. Because of the proven status of An. minimus as the primary vector of malaria in many areas of Thailand, solution of taxonomic problems in this group takes on added significance.

Ayurakitia: Only one species previously described for this genus and known to occur only in Northern Thailand. Specimens collected recently from pandanus axils in Southern Thailand belong to this genus but represent a new and undescribed species.

Culex: Culex (Culiciomyia) termi, this very unusual mosquito, the larvae of which have siphons longer than the combined length of head, thorax and abdomen, was described from Thailand by Thurman, 1955. Specimens were collected in Lampang Province during 1952-53. Since that time repeated efforts to collect this species by several subsequent investigators have been unsuccessful. Collectors of SMRL encountered this species several times in elephant foot-prints, the type habitat, along with three other new and undescribed species of Culex (Culiciomyia) in Mae Hongson Province during September.

Aedes: Aedes (Diceromyia), only one species of this subgenus had previously been reported from Thailand and prior to this report no collections of the immature stages had been made. The larvae of a new and undescribed species were collected several times from bamboo in the northern and southern provinces of Nan and Phangnga.

Summary: A large number of specimens were added to the collection during the year, including several not previously known from Thailand and others which are new but undescribed. Much additional information on the distribution and bionomics has been accumulated on many species. The Thai mosquito fauna is extremely complex and much remains to be learned, but after several years of collecting we are now at the point of understanding many of these complexities. An effort is underway to consolidate all available information on the Thai mosquito species. Detailed studies of several specific groups are continuing and several manuscripts are pending publication. Many of the early problems encountered in the study have been satisfactorily solved and prospects for even more accomplishments during the coming year look more promising.

Figure 1.

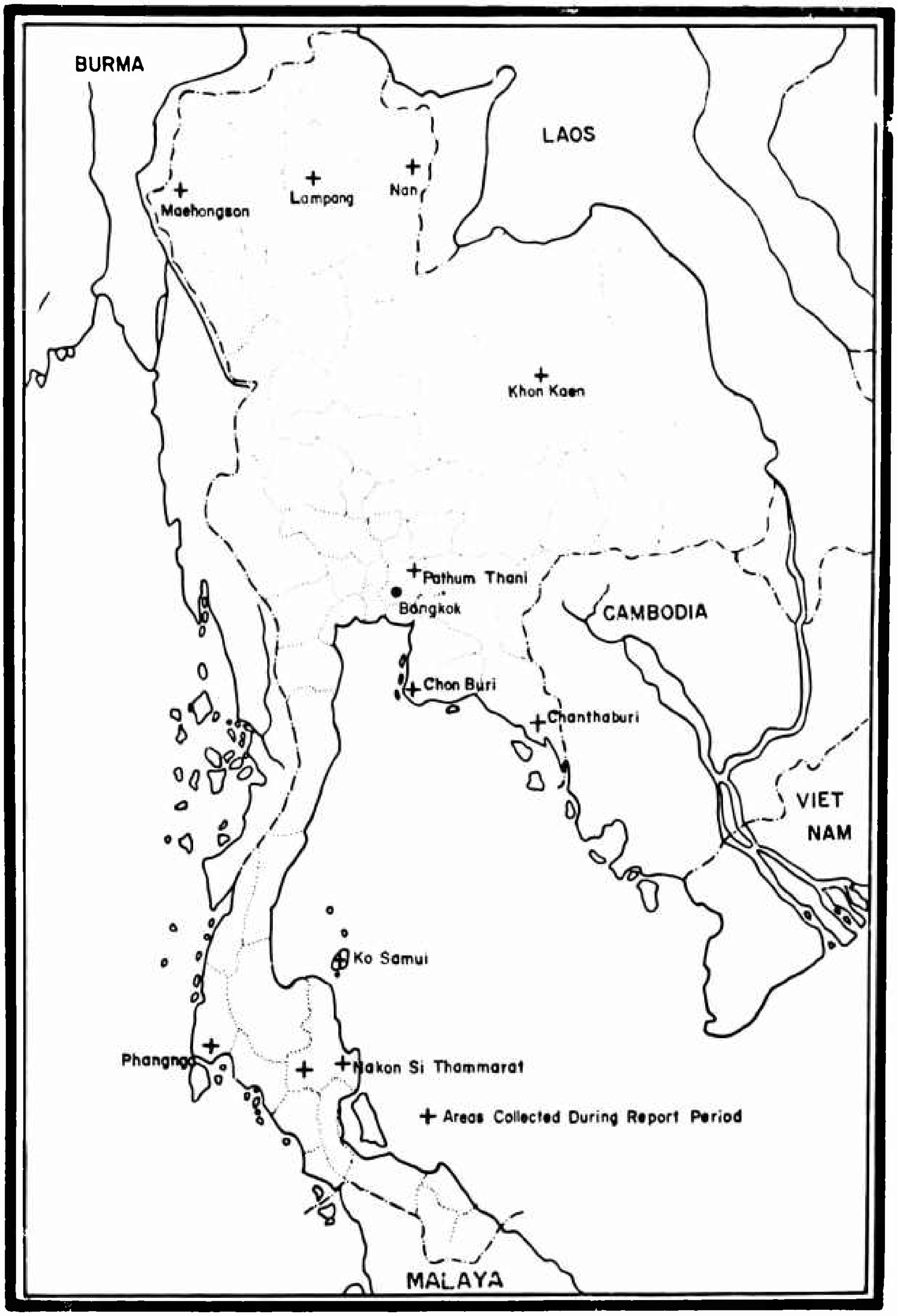
BURMA
LAOS
Maehongson
Lampang
Nan
Khon Kaen
Pathum Thani
Bangkok
CAMBODIA
Chon Buri
Chanthaburi
VIET
NAM
Ko Samui
Phangnga
Nakon Si Thammarat
+ Areas Collected During Report Period
MALAYA

Table 1A Tabulation of *Anopheles* larval collections made during the year.

SPECIES	HABITAT																						TOTAL	SHADE FREQUENCY		
	Stream Pool	Rock Pool	Stream Margin	Rice Field	Ditch-Canal	Ground Pool	Flood Pool	Wheel-Rut	Well	Seepages-Springs	Bog Marsh	Swamp	Elephant Foot-print	Animal Hoof-print	Stump Hole	Tree Hole	Fallen Tree Trunk	Pandanus Axil	Bamboo Stump	Split Bamboo	Crab Hole	Banana Stump	Total collections of each species.	None	Partial	Heavy
Number of each type habitat examined	47	65	14	19	44	15	44	17	7	8	21	6	106	4	16	105	25	12	105	64	15	2				
Anopheles (Anopheles)																										
An. annandalei interruptus																1							1			
An. asiaticus																				13			13			
An. barbirostris	2	2	1	3	4	3	4		1	2		1											23	8	11	4
An. barbumbrosus	10	2	3	1			3		1				1										21	1	11	9
An. bengalensis	26	3	9		1					5	2		[illegible]								2		49	3	18	28
An. campestris				1	25	1	1		5					1									34	2	27	5
An. crawfordi	[illegible]																						1			1

Table 1A (Cont.)

SPECIES	Stream Pool	Rock Pool	Stream Margin	Rice Field	Ditch-Canal	Ground Pool	Flood Pool	Wheel-Rut	Well	Seepages-Springs	Bog-Marsh	Swamp	Elephant Foot-print	Animal Hoof-print	Stump Hole	Tree Hole	Fallen Tree Trunk	Pandanus Axil	Bamboo Stump	Split Bamboo	Crab Hole	Banana Stump	TOTAL: Total collections of each species.	SHADE FREQUENCY: None	SHADE FREQUENCY: Partial	SHADE FREQUENCY: Heavy
Anopheles (Anopheles) Cont.																										
An. fragilis										1													1			1
An. hodgkini												1											1			1
An. hyrcanus group		1	2	4		2	2				1	1											13	9	2	2
An. indiensis				1			1				1												3	3		
An. insulaeflorum	3		1																				4			4
An. nigerrimus				2		1																	3	3		
An. palmatus	3		1																				4			4
An. peditaeniatus				2	1																		3	2	1	
An. roperi	1											1											2			2
An. sinensis			1	5							1	1											8	7		1
An. sintonoides															1	9	2	2	4	10		1	29		12	17

Table 1A (Cont.)

SPECIES	HABITAT																						TOTAL	SHADE FREQUENCY		
	Stream Pool	Rock Pool	Stream Margin	Rice Field	Ditch-Canal	Ground Pool	Flood Pool	Wheel-Rut	Well	Seepages-Springs	Bog-Marsh	Swamp	Elephant Foot-print	Animal Hoof-print	Stump Hole	Tree Hole	Fallen Tree Trunk	Pandanus Axil	Bamboo Stump	Split Bamboo	Crab Hole	Banana Stump	Total collections of each species.	None	Partial	Heavy
Anopheles (Anopheles) Cont.																										
An. tigertti																					2		2			2
An. umbrosus group									1			1											2			2
Anopheles (Cellia)																										
An. aconitus	1		1		7					1	1												11	3	7	1
An. annularis				1								1											2	2		
An. balabacensis	1	7	1				5	2		3	1		41	1									62	7	40	15
An. jamesil				1																			1	1		
An. kochi				3		1	2					1	3	1									11	7	2	2
An. maculatus	3	3	2	3			11			1	3		4										30	11	18	1
An. minimus			1	5							1												7	6	1	

Table 1A (Cont.)

SPECIES	HABITAT																						TOTAL	SHADE FREQUENCY		
	Stream Pool	Rock Pool	Stream Margin	Rice Field	Ditch-Canal	Ground Pool	Flood Pool	Wheel-Rut	Well	Seepages-Springs	Bog-Marsh	Swamp	Elephant Foot-print	Animal Hoof-print	Stump Hole	Tree Hole	Fallen Tree Trunk	Pandanus Axil	Bamboo Stump	Split Bamboo	Crab Hole	Banana Stump	Total collections of each specie..	None	Partial	Heavy
Anopheles (Cellia) Cont.																										
An. pampanai	1																						1	1		
An. philippinensis				6			1	1															8	6	2	
An. riparis macarthuri		3	1																				4	1	1	2
An. splendidus			1																				2	2		
An. subpictus				1	4	2			1		1												9	5	2	2
An. tessellatus				1	8				1														10		10	
An. vagus	1	3		11	9	3	5		1					2									35	19	15	1
Total species from each type habitat.	12	8	13	17	8	7	10	2	7	6	10	8	5	4	1	2	2	1	1	2	3	1				

Total collections from all type habitats for the year 937

Total collections made from all habitats listed 762

Total collections with Anopheles 285

Table 1B. Tabulation of *Anopheles* larval collections made during the year.

SPECIES	MONTH												ALTITUDE IN METERS							PROVINCE
	January	February	March	April	May	June	July	August	September	October	November	December	0–15	15–150	150–300	300–500	500–900	900–1400	1400–1650	Chanthaburi, Cholburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani, Surat Thani.
Anopheles (Anopheles)																				
An. annandalei interruptus						x									x					Nakorn Si Thammarat,
An. asiaticus						x				x				x	x					Nakorn Si Thammarat, Phangnga, Mae Hongson
An. barbirostris				x		x	x	x	x	x			x	x	x	x	x			Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani, Phangnga
An. barbumbrosus			x				x	x		x				x		x	x			Chanthaburi, Khon Kaen, Nan
An. bengalensis			x					x	x	x				x			x	x	x	Chanthaburi, Mae Hongson, Nan, Phangnga
An. campestris				x			x						x		x					Khon Kaen, Pathum Thani

Table 1B. (Cont.)

SPECIES	MONTH												ALTITUDE IN METERS							PROVINCE
	January	February	March	April	May	June	July	August	September	October	November	December	0–15	15–150	150–300	300–500	500–900	900–1400	1400–1650	Chanthaburi, Cholburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani, Surat Thani.
Anopheles (Anopheles) Cont.																				
An. crawfordi										x				x						Phangnga
An. fragilis										x				x						Phangnga
An. hodgkini										x				x						Phangnga
An. hyrcanus group						x	x	x						x	x	x	x			Khon Kaen, Nakorn Si Thammarat, Nan
An. indiensis						x				x				x						Nakorn Si Thammarat, Phangnga
An. insulaeflorum			x					x								x	x			Chanthaburi, Nan
An. nigerrimus						x		x						x	x					Nakorn Si Thammarat, Nan
An. palmatus			x														x			Chanthabur.
An. peditaeniatus				x				x					x	x	x	x	x			Phathum Thani, Nan
An. roperi										x				x						Phangnga
An. sinensis							x	x	x	x				x		x	x	x		Khon Kaen, Mae Hongson, Nan, Phangnga

Table 1B. (Cont.)

SPECIES	MONTH												ALTITUDE IN METERS							PROVINCE
	January	February	March	April	May	June	July	August	September	October	November	December	0–15	15–150	150–300	300–500	500–900	900–1400	1400–1650	Chanthaburi, Cholburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani, Surat Thani.
An. sintonoides			x			x				x				x	x	x	x			Chanthaburi, Nakorn Si Thammarat, Phangnga
An. tigertti			x														x			Chanthaburi
An. umbrosus group										x					x		x			Phangnga
Anopheles (Cellia)																				
An. aconitus				x		x		x		x			x	x	x		x			Khon Kaen, Nakorn Si Thammarat, Pathum Thani, Phangnga
An. annularis							x								x	x	x			Khon Kaen
An. balabacensis			x			x		x	x	x			x	x	x	x	x	x	x	Chanthaburi, Mae Hongson, Nakorn Si Thammarat, Nan, Phangnga
An. jamesii							x									x				Khon Kaen
An. kochi						x	x	x		x				x	x	x	x			Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Phangnga

Table 1. (Cont.)

SPECIES	MONTH												ALTITUDE IN METERS							PROVINCE
	January	Fabruary	March	April	May	June	July	August	September	October	November	December	0 15	15 150	150 300	300 500	500 900	900 1400	1400 1650	Chanthaburi, Choiburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani, Surat Thani.
Anopheles (Celiia) Cont.																				
An. maculatus		x				x	x	x	x					x	x	x	x	x		Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Surat Thani
An. minimus								x	x					x	x	x	x	x		Mae Hongson, Nan
An. pampanai								x								x				Nan
An. philippinensis						x	x	x						x	x	x				Khon Kaen, Nakorn Si Thammarat, Nan
An. riparis macarthuri						x								x		x				Nakorn Si Thammarat
An. splendidus									x									x		Mae Hongson
An. subpictus						x	x		x				x	x		x				Chanthaburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat
An. tessellatus				x									x	x						Pathum Thani
An. vagus				x		x	x	x	x				x		x	x	x			Cholburi, Khon Kaen, Mae Hongson, Nakorn Si Thammarat, Nan, Pathum Thani
Total Species		1	7	6	0	15	12	16	9	15			8	23	1	18	15	6	2.	

Table 2

Mosquito species collected during 1966–1967
not previously represented in SMRL collections.

SPECIES	PROVINCE
Aedes (Aedimorphus) culicinus, Edwards, 1922	Khon Kaen
Aedes (Diceromyia) u. sp.	Nan, Phangnga
Aedes (Subgenus Unknown) u. sp.	Nan
Anopheles (Anopheles) tigertii, Scanlon and Peyton, 1967	Prachinburi, Chanthaburi
Armigeres (Leicesteria) pendulus, (Edwards), 1914	
,, ,, traubi, Macdonald, 1960	Phangnga
,, ,, inchoatus, Barraud, 1927	,,
,, ,, u. sp.	,,
Ayurakitia u. sp.	Nakorn Si Thammarat, Phangnga
Culex (Culiciomyia) termi, Thurman, 1955.	Mae Hongson
,, ,, u. sp. 1	,,
,, ,, u. sp. 2	,,
,, ,, u. sp. 3	,,
Culex (Culex) litoralis, Bohart, 1946	Chanthaburi, Cholburi
Toxorhynchites (Toxorhynchites) amboinenensis, (Doleschall), 1857.	Nan

Publications.

Culex (Thaiomyia) dispectus, A New Subgenus and Species From Thailand (Diptera: Culicidae). Ralph A. Bram. Proc. Ent. Soc. Wash. 1966.

Illustrated Key To The Female *Anopheles* of Thailand. E.L. Peyton and J.E. Scanlon. Applied Scientific Rsch. Corp. Bangkok. 1966.

Six New Species of The *Culex (Lophoceraomyia) mammilifer* group from Thailand (Diptera : Culicidae). Ralph A. Bram and M. Rattanarithikul. Proc. Ent. Soc. Wash. 1967.

Anopheles (Anopheles) tigertti, A New Species of The *Aitkenii* Group from Thailand. J.E. Scanlon and E.L. Peyton. Proc. Ent. Soc. Wash. 1967.

An Annotated Checklist of The *Anopheles* of Thailand (*Diptera* : *Culicidae*) J.E. Scanlon, E.L. Peyton and D.J. Gould. In press.

2. Title: Insecticide Tolerance Level of Mosquitoes in Thailand.

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Objective: The development of resistance by mosquitoes to insecticides has posed a considerable threat to the success of public health efforts in combating mosquito-borne disease in many parts of the world. In Thailand, insecticides have been in use for several years against agricultural pests and more commonly in malaria eradication programs. The extent of resistance among mosquitoes in this country cannot be adequately assessed because relatively little information is available on the status of susceptibility of these species to insecticides. Therefore, this study is designed to determine the susceptibility of mosquitoes to insecticides with emphasis on vectors or potential vectors of human disease and to assess the possible consequences of resistance, whether physiological or behavioristic, on the disease incidence.

Progress: This report covers results obtained from (1) studies conducted by SMRL on the susceptibility of local mosquitoes to insecticides in current use and from (2) collaborative studies, with personnel of USDA and WRAIR, on field evaluation of newer insecticides and repellents.

I. SUSCEPTIBILITY OF MOSQUITOES TO INSECTICIDES.

The susceptibility levels of nine species of mosquitoes to DDT and/or dieldrin have been determined. These species included three culicines, *Aedes aegypti*, *Culex gelidus* and *C. tritaeniorhynchus* and six anophelines, *Anopheles balabacensis*, *An. maculatus*, *An. minimus*, *An. splendidus*, *An. tessellatus* and *An. vagus*. All of the species tested are known to feed freely on man. They have also been, except for *An. splendidus*, *An. tessellatus* and *An. vagus*, incriminated in the transmission of human disease in this country and elsewhere.

Material representative of all species tested was obtained from one or more of the following localities: the Experimental Farm of Kasetsart University in the Bang Khen District of Bangkok and from the Provinces of Saraburi, Cholburi, Nakornrajsima and Surathani. Dwellings in these Provinces have been treated for several years, under the malaria eradication program, with DDT residual sprays for one or two cycles per year. At the Kasetsart Experimental Farm, several insecticides ranging from parathion and chlorinated hydrocarbons to Paris green have been in continuous use. Specific locations for any single species tested will be mentioned under its appropriate section in the discussion. *Anopheles balabacensis* was the only species tested with material provided from a laboratory colony maintained by SMRL. The standard WHO

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adult and larval tests were used throughout, except when it was necessary to use the "time in concentration" technique to demonstrate low levels of resistance. Observed mortalities of mosquitoes obtained in the various tests were corrected for control mortality using Abbott's formula. Log dose probit mortality regression lines were constructed and the method of Lichfield and Wilcoxon was followed for the calculation of the limits of confidences.

Culex gelidus. Susceptibility tests on the adults showed that this species had developed an intermediate resistance to DDT and was completely resistant to dieldrin at Bang Khen District of Bangkok while it was still susceptible to DDT (the only insecticide tested) at Bang Phra, Cholburi Province. Resistant adults from Bang Khen showed a two-fold increase of the LC_{50} of DDT and from two to six-fold increase of LC_{90} value over the susceptible individuals from Bang Phra (Table I). Also the slope of the ld-p line as shown in Figure I, 6 was steeper for the susceptible adults than those constructed for the resistant ones of Bang Khen.

The appearance of resistance to DDT was first detected when 13% of the adults survived exposure to the highest concentration (4%) of the toxicant in the initial test (1). The percentage of survivors not only increased, in the follow up test (no. 2) at the 4% DDT concentration but an increase in the LC_{50} and LC_{90} values was also recorded. This resulted in a shift in the position of the dosage mortality line without change in slope indicating-in addition to increased resistance-an increase in vigour tolerance of the population tested (Fig. I, 1&2). Later tests conducted on populations of this species from Bang Khen continued to show the presence of resistant individuals though their percentages fluctuated from time to time (Table I, 3, 4&5).

Resistance of adults of C. gelidus from Bang Khen to dieldrin was discovered when this species was recently tested. Table 2 shows that approximately 68% of the adults survived one hour exposure to 4% dieldrin, and when adults were exposed to the same concentration for a two hour period, 47% of the total number tested survived through the 24 hours holding following exposure.

It is interesting to note that larvae tested from Bang Khen, where the resistant adults were found, continued to be susceptible to DDT. The LC_{50} value was 0.0085 ppm of DDT when tested in March 1967 (Fig. II, 2) compared with 0.0089 ppm obtained earlier in November, 1965 (Fig. II, 1). Larvae were also found susceptible to both dieldrin and BHC during 1965 with LC_{50} values of 0.010 and 0.24 ppm respectively. The dosage-mortality regression lines in Figure II show a slight increase in tolerance of the larvae to dieldrin and BHC than to DDT.

The absence of resistance among larvae and its appearance among adults from the same locality suggested the further testing of these larvae by the "time-in-concentration" technique. The reliability of the WHO test for resistance in larval populations, especially where resistance is not pronounced, has been open to criticism, and the "time-in-concentration" technique has been substituted in these studies for detection of resistance in larvae. Late fourth instar larvae were exposed to discriminating dosages of 8 ppm of dieldrin and 2.5 ppm of DDT. Mortality counts were made every 15 minutes after the larvae were placed in the test solutions. The percentage mortality versus time were plotted on log probit paper and the results are shown in Figure III.

In the dieldrin test the regression lines A and B reflected an almost identical genetic constitution of the population involved. The pronounced inflections of the regression lines clearly indicated the presence of two genotypes. Each regression line showed a plateau distinguishing the homozygous susceptible from the heterozygous resistant larvae. In this test approximately 72% of the larvae were susceptible and 28% were heterozygous resistant to dieldrin.

The regression line constructed for the DDT test also showed a pronounced inflection indicating the presence of two phenotypes. While 98% of the larvae appeared to be homozygous susceptible, the presence of heterozygous resistant larvae, though at a low level, was indicated.

Table 1. Results of DDT susceptibility tests on adult <u>Culex gelidus</u> from Bang Khen and Bang Phra (1966-1967)

Test No.	Date	Per cent mortality at each concentration 0.25%	0.5%	1%	2%	4%	Per cent control mortality	LC_{50}	95% confidence limits of LC_{50}	LC_{90}	Slope
				Bang Khen							
1	30Mar.- 5 Apr. 66	15(104)	27(105)	29(105)	61(105)	87(105)	5(105)*	1.23%	1.03-1.46	6.75%	1.73
2	28Apr.- 4May66	7(70)	23(70)	42(70)	36(70)	84(70)	0(70)	1.59%	1.29-1.96	8.41%	1.77
3	10-12 May66	8(61)	17(60)	32(60)	50(59)	86(60)	2(60)	1.49%	1.19-1.87	6.61%	1.98
4	27May66	6(79)	21(77)	27(80)	57(80)	96(75)	1(79)	1.19%	1.003-1.41	3.81%	2.54
5	Mar.67	3(139)	6(140)	23(139)	69(136)	94(137)	4(138)	1.40%	1.22-1.60	3.81%	2.96
				Bang Phra							
6	Feb.67	7(80)	35(80)	79(80)	81(80)	100(80)	14(80)	0.66%	0.58-0.75	1.41%	3.89

* Figures shown in parenthesis represent the number of adults tested at each concentration.

Table 2.

Results of dieldrin susceptibility tests of adult C. gelidus from Bang Khen District, Bangkok, March 1967

Exposure time	Per cent mortality at each concentration							Per cent control
	4	1.6	0.8	0.4	0.2	0.1	0.5	mortality
1 hour	32(91)*	19(92)	17(92)	5(97)	0(85)	0(96)	0(98)	1(97)
2 hours	53(20)	10(21)	–	–	–	–	–	5(20)

* Figures shown in parenthesis represent number of adults tested.

It may be concluded from those results that adults of C. gelidus from Bang Khen, showed intermediate resistance to DDT but were completely resistant to dieldrin. The appearance of this resistance was also confirmed by the "time-in-concentration" technique through which two genotypes were recognized. The development of this physiological resistance is probably the result of selection pressure of a high order imposed on the population through the extensive use of insecticides in the area.

Culex tritaeniorhynchus. Only the adults of this species were tested for susceptibility to DDT. Adults were collected while biting cattle in the vicinity of houses near the Botanical Gardens at Saraburi. In addition, larval material was obtained for testing from Kasetsart's Experimental Farm at Bang Khen, Bangkok. These were reared through in the insectary and the sugar-fed females were tested when 4-5 days old. The LC_{50} for adults from Saraburi was 0.50% with 95% confidence limits of 0.4-0.6% and an LC_{90} of 1.8%. The LC_{50} for the Bang Khen adults was 0.57% with C.L. of 0.5-0.7% and an LC_{90} of 1.05%. Based on the LC_{50} values this species appeared to be equally susceptible to DDT at both locations. The high LC_{90} value determined for Saraburi adults resulted in a slightly flat dosage-mortality regression line (slope 2.05) indicating a heterogenous population (Fig IV). The steep line (slope 4.74) constructed for Bang Khen adults indicated a homogenous susceptible population. This reflected the uniformity in age and nutritional conditions of these adults in contrast with those wild collected females from Saraburi.

Aedes aegypti Resistance of Aedes aegypti to DDT has been reported from differant areas of Thailand during the past few years. However, interest in the determination of its susceptibility status from Surathani Province arose following an epidemic of Thai Hemorrhagic Fever on the island of Koh Samui in that province. Eggs were collected from Ang Thong and Taling Ngam on the island of Koh Samui and the neighbouring islands of Koh Phaluai and Koh Phangan. Larvae were later tested in the laboratory against DDT. Resistance was detected in larvae representative of all locations considered. Larval mortality after 24 hours exposure to 2.50 ppm of DDT ranged from 63% at Ang Thong to a low level of 22% at Koh Phaluai (Table 3).

Larvae from three locations were also tested for resistance to dieldrin by the "time-in-concentration" technique. Larvae were exposed to an established discriminating dose of 5 ppm dieldrin and mortality was observed at hourly intervals over a 24 hours period. Results obtained by this method are expressed by the regression lines 1, 2 and 3 in figure V. These lines show the presence of three distinct genotypes in each of the samples tested. Each regression line shows a pronounced inflection and the presence of a plateau distinguishing the homozygous susceptibles from the heterozygous resistant larvae. The proportion of susceptible larvae was lowest (53%) at Koh Phangan and highest (80%) at Ang Thong. The survival of 3.8%, 17.8% and 6.3% of larvae from Ang Thong, Koh Phangan and Taling Ngam, respectively, to this discriminating

dose constituted the proportions of the homozygous resistant larvae present. The remaining proportions were the heterozygous resistant larvae. It was concluded that larvae of Aedes aegypti are resistant to both DDT and dieldrin at all locations tested from Surathani Province.

Table 3.

Results of DDT susceptibility tests on Aedes aegypti larvae from Surathani Province, 1967.

Concentration (ppm)	Percent larval mortality at each location			
	Koh Phaluai	Taling Ngam	Koh Phangan	Ang Thong
2.50	22 (60)*	32 (60)	30 (60)	63 (79)
0.5	2 (60)	2 (59)	0 (60)	8 (78)
0.1	0 (60)	0 (60)	0 (60)	0 (80)
0.02	0 (60)	0 (60)	0 (60)	0 (80)
0.004	0 (60)	0 (60)	0 (60)	0 (78)
Control	0 (80)	0 (80)	0 (80)	1 (79)

* Figures shown in paranthesis represent the number of larvae tested at each concentration.

Anopheles balabacensis The susceptibility level of adults of this species to DDT was established previously with material collected at Kao Mai Kaeo, Cholburi Province. Since no information was available on its larval susceptibility, larvae from the SMRL laboratory colony were tested for this purpose. The LC_{50} was 0.015 ppm indicating the susceptibility of the larvae to this insecticide. It is interesting to note the small slope value (Table 4) of the mortality-regression line (Fig VI) obtained reflecting the heterogenity of the larvae of this colonized strain.

Anopheles maculatus.—Adults of this species were tested for their susceptibility of DDT and dieldrin. Blood-fed females were collected while biting man at Kho Mai Kaeo, Cholburi Province during February 1967. Some adults were held over for egg laying and as a result larval testing against DDT was possible. Results showed an LC_{50} of 0.056% of dieldrin with 95% confidence limits of 0.042-0.075%; an LC_{90} of 0.1% and a slope value of 4.32. These values indicated that adults of this species are highly susceptible to dieldrin.

In the DDT tests the LC_{50} values of 0.65% and 0.010 ppm obtained for the adults and larvae respectively pointed to the susceptibility of this species to this insecticide. The LC_{50} for the adult maculatus was two times greater than that (0.3%) determined for adult balabacensis tested from the same area by other workers. Whether this small amount of tolerance is interspecific or brought about by insecticide pressure is difficult to say. In the meantime, it was surprising to find that the LC_{50} of 0.010 ppm determined for maculatus larvae was the lowest recorded for any of the anopheline species tested (Table 4.)

Anopheles minimus Both adults and larvae of this species were tested for their susceptibility to DDT. Blood-fed females were collected biting man in the vicinity of houses near the Botanical Gardens at Saraburi, Saraburi Province. Eggs were also recovered from some of the adults to support larval testing. The LC_{50} value of 0.31% is the lowest obtained for any of the adult anophelines tested, and falls in the range of normal

Table 4.

Results of DDT susceptibility tests on some Anopheles species from Thailand.

Species	Date tested	Locality	Stage tested	LC_{50}*	95% Confidence limits of LC_{50}	LC_{90}	Slope
Anopheles balabacensis	May, 66	Laboratory colony	L	0.015	0.013–0.018	0.052	2.42
An. maculatus	Feb., 67	Kao Mai Kaea, Cholburi Province	A	0.65	0.51–0.84	1.35	3.86
		,, ,,	L	0.010	0.005–0.021	0.023	3.63
An. minimus	Jan., 67	Botanical Gardens, Saraburi Province	A	0.31	0.25–0.37	0.49	6.41
		,, ,,	L	0.016	0.0136–0.0198	0.036	3.82
An. splendidus	Feb., 67	,, ,,	A	0.48	0.42–0.56	0.86	5.18
An. tessellatus	Dec., 66	,, ,,	L	0.012	0.006–0.025	0.026	3.76
An. vagus	May, 67	I Bang Khen District, Bangkok	A	0.99	0.86–1.16	2.82	2.82
		II Mo Ban Takhop near Pak Thong Chai, Nakornrajisima Province	A	0.97	0.81–1.16	3.01	2.61

* LC values in ppm for larvae and in per cent for adults.

susceptible anopheles. The steep mortality regression line with a slope value of 6.41 also indicates that these adults are homogenous susceptible to DDT (Fig. VII) Although the LC_{50} of 0.016 ppm was the highest obtained among the anopheles larvae tested, the minimus larvae were still susceptible to DDT.

Anopheles splendidus. Adults of this species were collected at the same location as those of minimus, and they were also tested for susceptibility to DDT. An LC_{50} of 0.48% indicated the susceptibility of this species to DDT. The confidence limits of the LC_{50}, LC_{90} and the slope values are given in Table 4.

Anopheles tessellatus: Only the larvae of this species were tested for their susceptibility to DDT. The few blood-fed females collected at Saraburi were held in the insectary to provide eggs for the eventual testing of the larvae. The LC_{50} of 0.012 ppm showed that this species is also susceptible to DDT in the larval stage.

Anopheles vagus. Adults of this species obtained from two different sources were tested for their susceptibility to DDT. Females were collected while biting cattle at Kasetsart University Farm, in Bang Khen District of Bangkok and while biting man at Mo Ban Takop near Pak Thong Chai, Nakornrajisima Province. Almost identical LC_{50} values (0.97 and 0.99%) were obtained for this species at both locations. These values are greater than those obtained for the other susceptible anophelines tested (Table 4.). The slope of the regression line is flatter than those of the other species indicating considerable heterogeneity of vagus population (Fig. VII). Tests revealed the presence of 6% survivors to the highest DDT concentration (4%) in one of four tests made with adults from Mo Ban Thakop. Also in one of the replicates run with adults from Bang Khen, 10% of the mosquitoes survived exposure to the same high concentration. With this proportion of survivors, these tests indicate the presence of an intermediate level of resistance among population of this species, and suggest the possibility that we are dealing with a heterogenous population part of which is DDT resistant.

II COLLABORATIVE STUDIES ON INSECTICIDES AND REPELLENTS.

1. Laboratory larvicide tests:

Six insecticides, namely Abate, Dursban, fenthion, naled, malathion and baygon were tested against wild-collected larvae of Culex gelidus; malathion, baygon and naled were also tested against C. tritaeniorhynchus.

The calculated LC_{50} and LC_{90} values are given in table 5. These results showed that abate, dursban and fenthion were very effective against C. gelidus larvae. Based on the LC_{90}, Abate was 90 times, dursban was 24 times and fenthion was 12 times more toxic than the malathion standard. Naled was about as toxic as malathion, but Baygon was only one-fifth as toxic as this standard insecticide. Malathion was more toxic than Naled and Baygon against C. tritaeniorhynchus larvae.

Table 5.

Results on toxicity of insecticides to larvae of two Culicine species from Bang Khen District, Bangkok.

Insecticide	Culex gelidus		C. tritaeniorhynchus	
	LC_{50}	LC_{90}	LC_{50}	LC_{90}
Abate	.00012	.00023	—	—
Dursban	.00045	.00076	—	—
Fenthion	.00092	.0016	—	—
Malathion	.0108	.017	.0027	.012
Naled	.011	.022	.0038	.0073
Baygon	.058	.182	.054	.082

2. Fogging tests against a natural mosquito population:

Tests were conducted in the Klong Toey area of Bangkok to evaluate the effectiveness of fogs containing 1%, 2% or 4% malathion in diesel fuel in controlling mosquitoes, principally Culex quinquefasciatus. Results, presented in Table 6, showed that the mosquito population was reduced from 67% to 74% below the pretreatment counts in all the treatments. Of the mosquitoes collected the night of the treatment, 95% of these exposed to the 1% malathion died, and 99% of those exposed to the 2% and 4% malathion fogs were killed.

Table 6.

Results of malathion fogging tests in Bangkok against Culex quinquefasciatus.

Insecticide (%)	No. mosq/man/hr. Pretreatment	No. mosq/man/hr. Posttreatment	% Reduction	% mortality of captured mosquitoes after 24 hrs.
1	51	13	74	95
control	67	43	36	6
2	83	23	65	99
control	45	49	0	18
4	65	13	71	99
control	25	29	0	10

3. Toxicity of Abate and Malathion to fresh–water fish.

Tests were conducted in the laboratory to determine the toxicity of Abate and malathion to two species of edible fish, Cyprinus carpio and Tilapia mossambica, in rice fields and klongs. Both materials are of low mammalian toxicity and lend themselves to future mosquito control operations. Specimens of both

species were exposed for 24 hours to klong water containing concentrations of 10, 1. 0.1 and 0.01 ppm of the two chemicals, respectively. No kill was obtained except at the 10 ppm dosage of malathion against T. mossambica. In a second test, each species was tested in concentrations of Abate at 100 and 1,000 ppm. The higher concentration killed all the fish in 15 minutes and the lower killed them all in 2 hours.

4. Toxicity of Abate to fresh-water shrimp. A laboratory test was conducted to determine the toxicity of Abate to the edible shrimp (Caridina sp.) also commonly stocked in rice fields and klongs. Shrimps were tested in klong water containing Abate at concentrations of 0.01, 0.1, 1.0, and 10.0 ppm. Mortality counts taken after 24 hours gave the following results:

Dosage (ppm)	Per cent mortality
0.01	40
0.1	0
1.0	y0
10.0	90
0 (control)	0

5. Toxicity of Abate Aedes aegypti larvae in concrete water jugs.

Abate was tested in different formulations and concentrations, against Adedes aegypti larvae in concrete water storage jugs. Each jug was filled with 45 gallons of water into which the insecticide was added to give the final concentration required. Table 7 gives a list of all the concentrations of the different formulations used. Fifty late third-or early fourth-instar larvae collected from the wild were introduced once a week into each jug and checked for mortality 3 days later. If larvae were present in any jug on two consecutive weeks, such a treatment was considered no longer effective. Two parrallel series were run simultaneously, one in which the water level was maintained constant ("Static") while in the other ("Fluctuating") 15 gallons of wates were dished out and replaced with fresh water every week to simulate normal usage. This test was run in triplicate with three jugs of fresh water as controls.

Table 7.

Toxicity of Abate to Aedes agypti larvae in concrete water jugs.

Formulation	Concentration (ppm)	Condition	Ave. weeks of effective residual toxicity
Emulsifiable concentrate	1.0	Static	> 34
	1.0	Fluctuating	> 34
	0.1	Static	19.0
	0.1	Fluctuating	16.3
	0.05	Static	20
	0.05	Fluctuating	13.7
	0.025	Static	15
	0.025	Fluctuating	11
Granules	1.0	Static	> 34
	1.0	Fluctuating	> 34
	0.1	Static	22.5
	0.1	Fluctuating	14.3
Impregnated concrete pellet	?	Static	11.7
	?	Fluctuating	8.3

Results obtained are expressed as the average numbers of weeks of effective residual toxicity for each treatment (Table 7). Abate when used as emulsifiable concentrate or as granules at a concentration of 1.0 ppm, gave an exceptionally long residual effect lasting over 34 weeks in both the static and fluctuating series. It is interesting to mention that the emulsifiable concentrate of Abate gave an effective control against aegypti for 11 weeks at the low concentration of 0.025 ppm in the fluctuating series. The least effective formulation was that of the impregnated pellet, which gave an effective treatment lasting for only 11.7 weeks in the static series versus 8.3 weeks in the fluctuating ones.

6. Field tests with mosquito nets treated with repellents.

Tests were conducted on the evaluation of mosquito nets made of 1/4-inch mesh netting and treated with two repellents against Culex quinquefasciatus and Aedes aegypti in the Klong Toey area of Bangkok. Nets were treated with Deet and a mixture of M-1960 at the rate of 0.5 gram of repellent to 1 gram of netting. M-1960 consists of 30% benzyl benzoate, 30% N-butyl-acetanilide, 30% 2-butyl-2-ethyl-1, 3-propanediol, and 10% emulsifier. Nets were hung in selected houses and two subjects sat inside each of the treated and untreated nets for two hours. The mosquitoes that entered were collected and identified. One set of nets was tested between 1300 and 1500 hours, the time when Aedes aegypti was active, while the other set was checked between 1900 and 2100 hours when Culex quinquefasciatus was most prevalent. Results indicated that the nets treated with M-1960 gave complete protection from Aedes aegypti for 98 days versus 91 days by the Deet treated nets. M-1960 treated nets gave also complete protection from Culex quinquefasciatus for 126 days versus 113 days by the one treated with Deet. Another set of nets was also tested in forested areas south of Pak Thong Chai, Nakornrajisima Province. The anopheline population was low at the time, and only two Anopheles balabacensis entered the untreate net while none were collected in the treated ones.

7. Detection of Deet-treated subjects under jungle conditions.

Tests were conducted to determine whether Thai men and women could detect subjects treated with Deet when concealed in jungle bush. This test was run in a tropical evergreen forest south of Pak Thong Chai. Five Thai men and two Thai women served as detectors and nine Thai men and one American served as subjects. Each subject was treated with 3 ml. of deet, alcohol or water applied to their arms. Treated subjects were then stationed individually 5 or 10 feet from a trail, or in groups of five (all treated with the same material) 8 feet from the trail. Each detector walked down the trail and recorded the odor of deet at each numbered position at which it was detected. Replicate tests were made in the morning, afternoon, and night.

In the combined morning and night tests (when the humidity was high), with subjects located 10 feet from the trail, the male detectors made correct positive identification of deet 13% of the time (i.e., at 9 of 70 positions where the subjects were actually treated with deet) and false identification of deet 6% of the time (i.e., at 8 of 130 positions where the subjects were treated with alcohol or water). Results given in Table 8 showed that correct positives always exceeded false positives, except in the afternoon tests. However, when these detectors were allowed to smell treated arms the male detectors made 83% correct positive and 20% false positive determinations versus 54% correct positive and 8% false positive determinations by the female detectors.

Table 8.

Results of tests on the detection of deet under jungle conditions.

Test conditions	Male detectors Correct positives	False positives	Female detectors Correct positives	False positives
Morning & night tests				
Individual subjects at				
10 ft from trail	13	6	7	2
5 ft from trail	20	6	14	5
Groups of subjects at				
5 ft from trail	25	5	37	1
Afternoon tests				
Individual subjects at				
10 ft from trail	0	1	0	0
5 ft from trail	1	1	0	0
Groups of subjects at				
5 ft from trail	5	5	0	0

Summary: The susceptility status of nine species of mosquitoes from various localities in Thailand to DDT and/or dieldrin has been established. Adult Culex gelidus from Bang Khen, Bangkok showed intermediate resistance to DDT but were completely resistant to dieldrin. Aedes aegypti larvae from Suratani Province were also found resistant to both insecticides. The appearance of resistance among populations of these two species was confirmed by the "time in concentration" technique by which two and three genotypes were recognized for C. gelidus and A. aegypti respectively. The possibility that Anopheles vagus adults have developed resistance to DDT at two localities is suspected on basis of the appreciable increase observed in the LC-50 value and presence of survivors following exposures at the highiest concentration of 4% DDT. Adults and/or larvae of C. tritaeniorhynchus, Anopheles balabacensis, An. maculatus. An. minimus, An. splendidus, and An. tessellatus tested from different sources were found still susceptible to DDT.

Other studies on the evaluation of insecticides and repellents were conducted in collaboration with members of the USDA and WRAIR. These included testing of some promising insecticides for toxicity against larvae of three local mosquito species and to edible fresh water fish and shrimp. Fogs containing malathion were tested against natural mosquito populations and produced significant reduction in the populations. Special bed nets treated with repellents were effective against two species of mosquitoes for more than three months.

Fig. 1 Dosage-mortality regression lines of DDT against adult *Culex gelidus* from Bang Khen and Bang Phra.

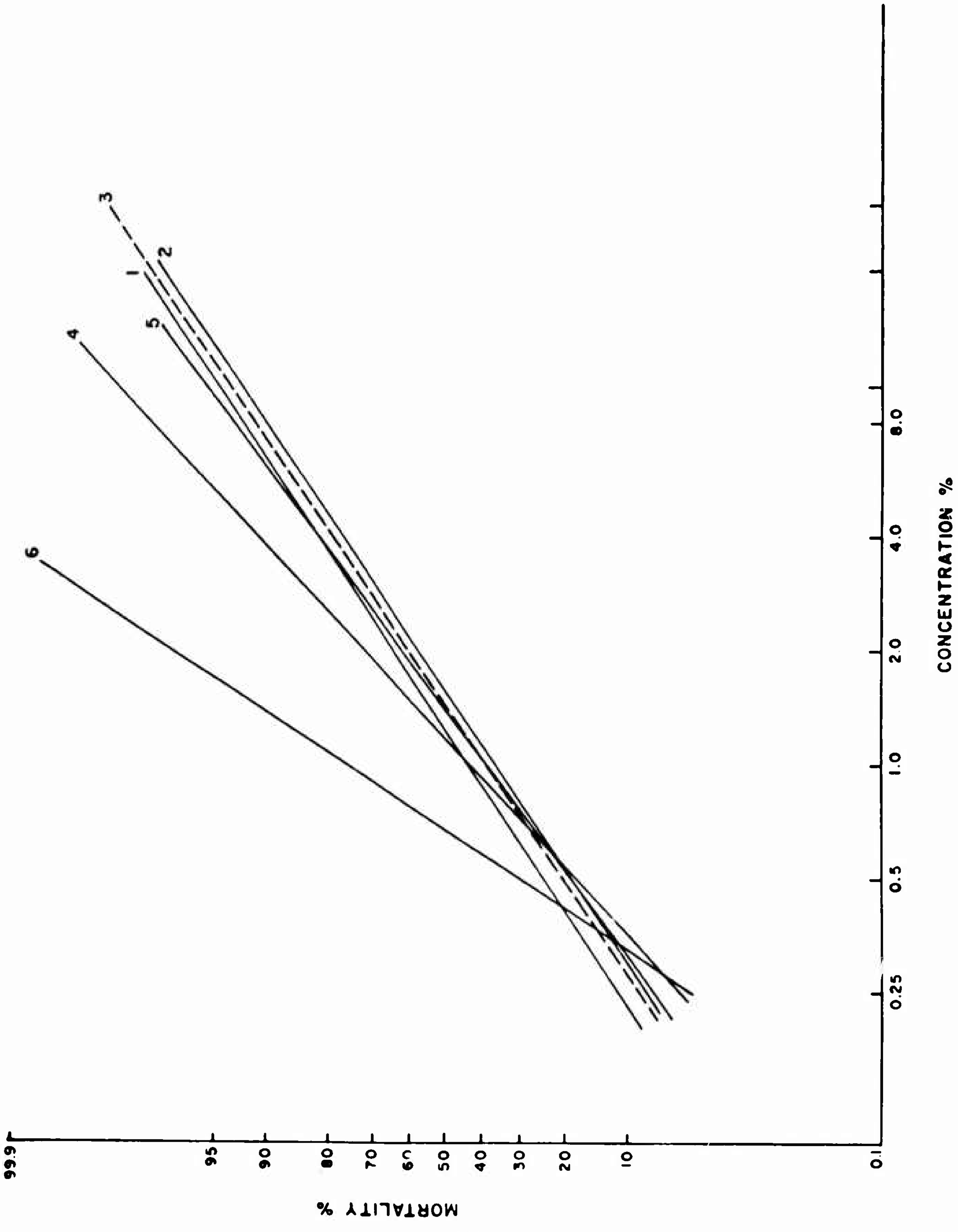


Fig. II Dosage-mortality regression lines of some chlorinated hydrocarbons against larvae of *Culex gelidus* from Bang Khen District, Bangkok.

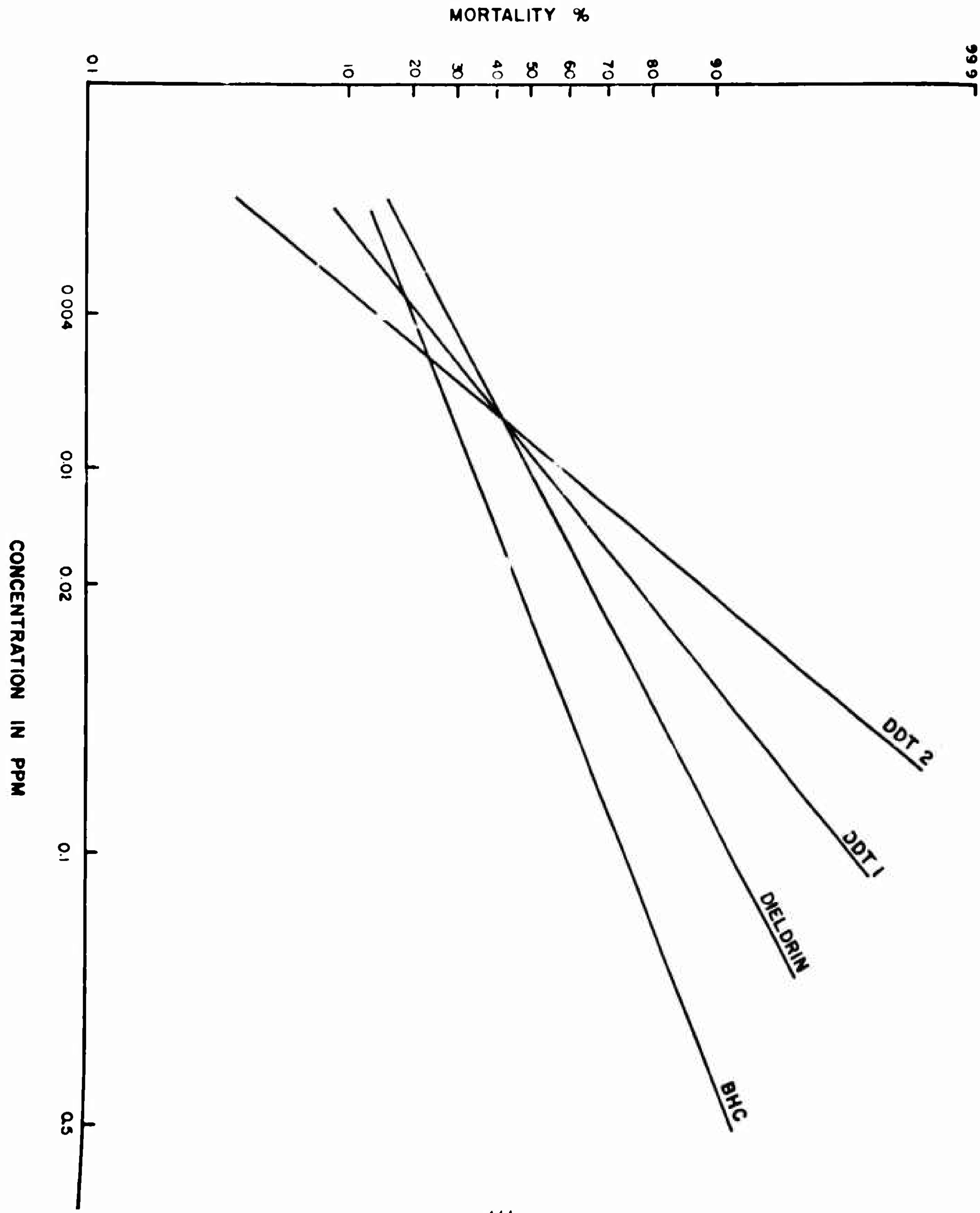


Fig. III Mortailty regression lines of DDT and dieldrin when tested against Culex gelidus larvae by the "Time In Concentration" technique.

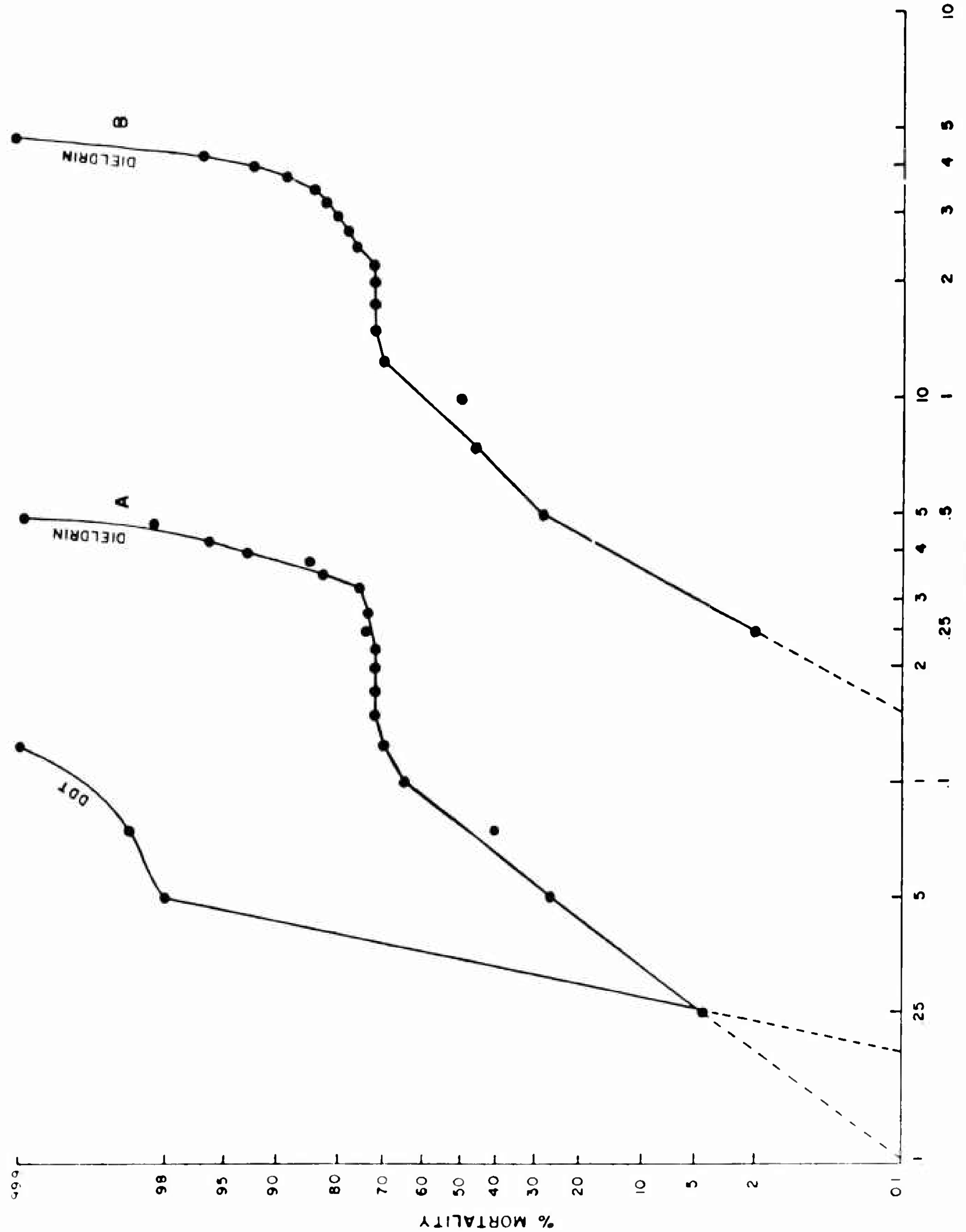


Fig. IV Dosage-mortality regression lines of DDT against adult Culex tritaeniorhynchus.

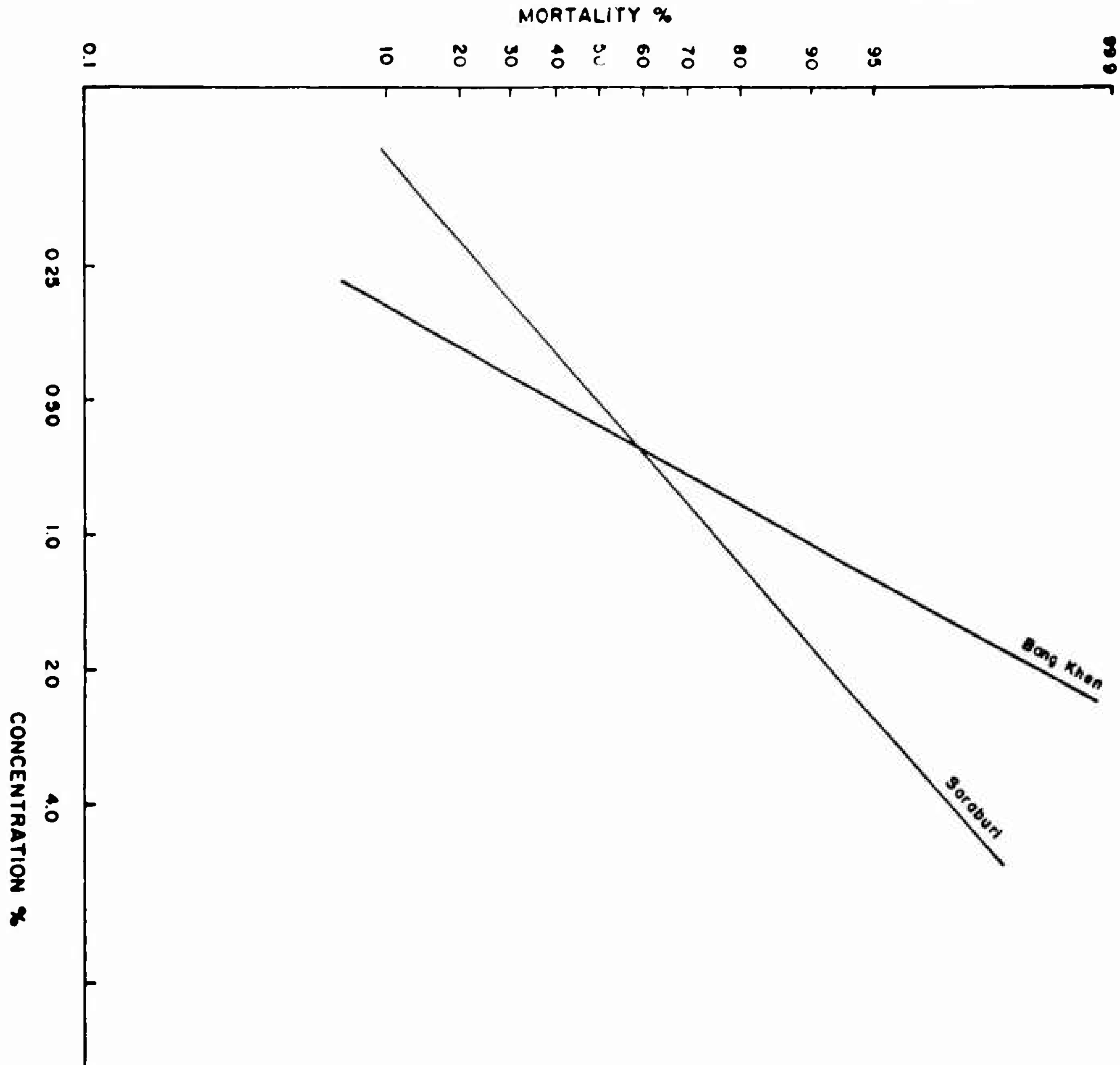


Fig. V. Mortality regression lines of dieldrin against Aedes aegypti larvae when tested by the "Time In Concentration" technique.

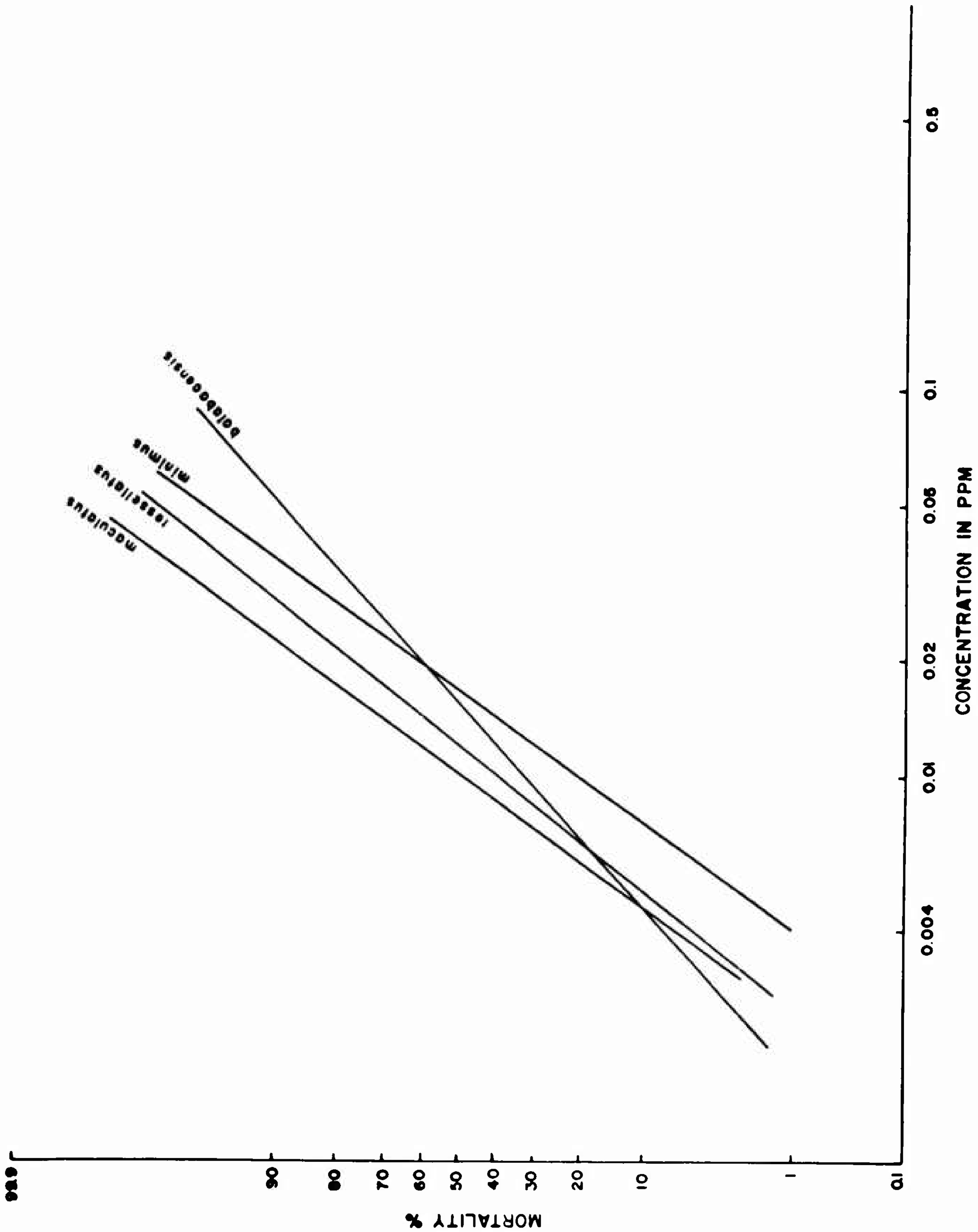


Fig. VI Dosage-mortality regression lines of DDT against larvae of some anopheles species.

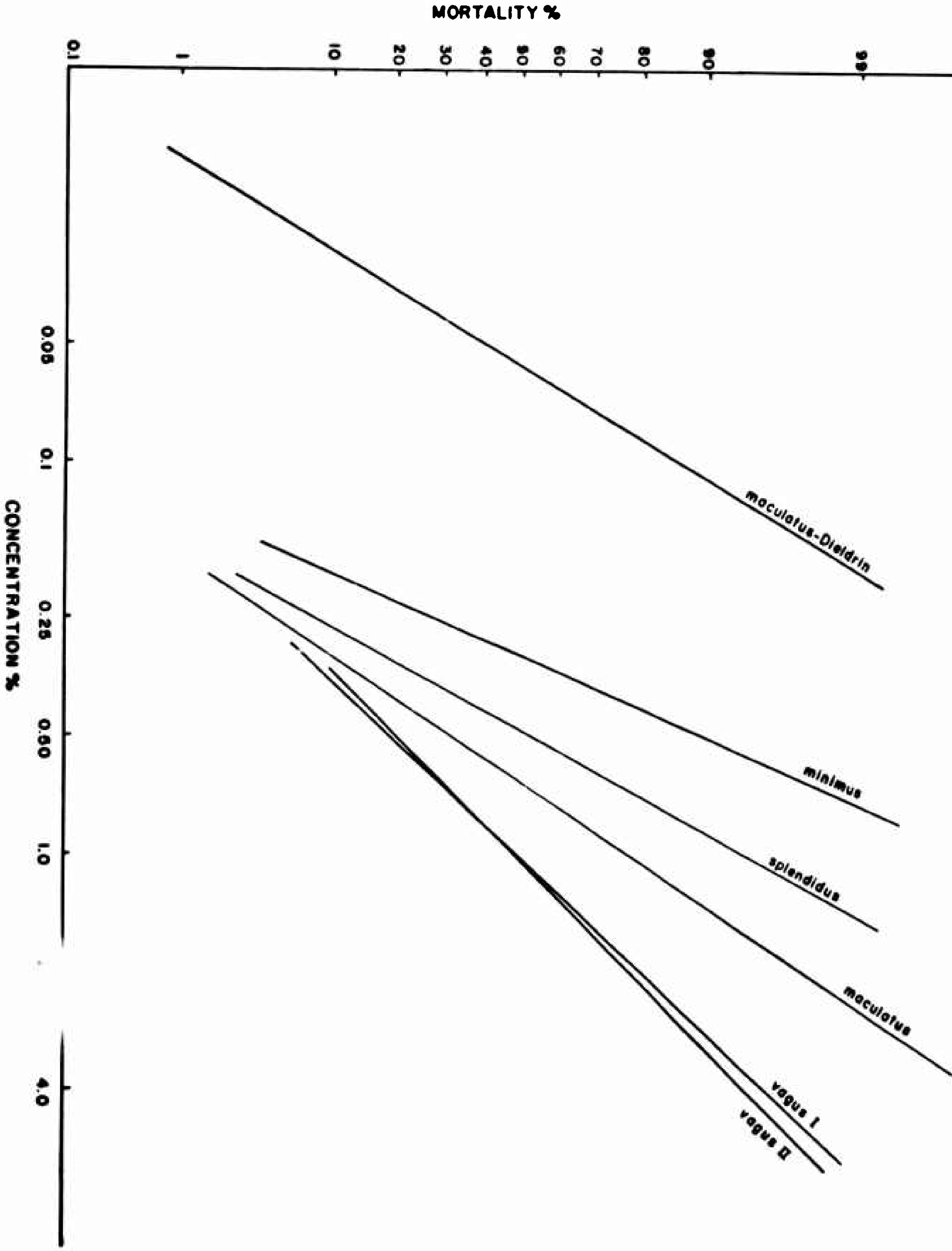
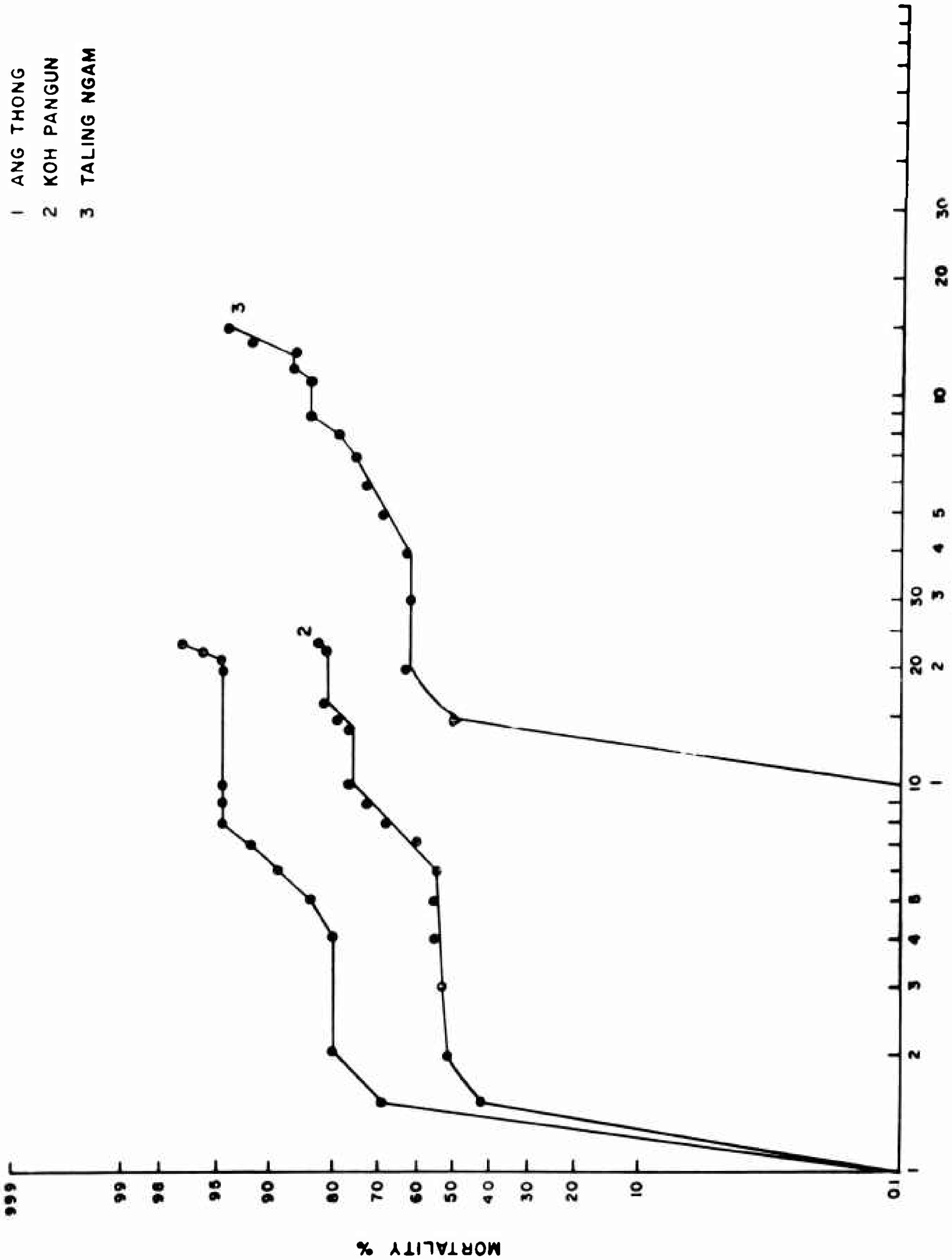


Fig. VII Dosage-mortality regression lines of DDT and dieldrin against adults of some anopheles species.



3. Title: Feeding habits of mosquitoes of medical importance in Thailand.

Principal Investigator: Moufied A. Moussa, Major, MSC

Associate Investigator: Pacharee Nawarat, B.S.

Objective: The host range of a mosquito vector is one of the important factors affecting the persistence and spread of mosquito-borne diseases. The vectorial capacity of a species depends, among other factors, on its preference for human blood and the frequency of its contact with man. The purpose of this study is to determine the natural hosts of the medically important species in Thailand in support of various mosquitoborne disease studies in progress.

Description: Blood engorged mosquitoes were collected during the course of studies on malaria and arthropod-borne viruses. Saline extracts of gut contents were tested by the precipitin ring-test and the agar-gel diffusion technique for reactions against a battery of antisera produced in chickens and rabbits against human, monkey, cow, buffalo, dog, pig, chicken, horse, and rat sera.

Progress: Problems with broad cross-reactivity have been encountered with some of the antisera when tested with heterologous antigens in the precipitin ring test. Consideration was given to replacing the ring test with the agar-gel diffusion technique. When parallel tests were run with both techniques using the same antisera and mosquitoblood meal extracts, wide cross reactions were obtained in the ring test with most of the antisera, while none occurred in the agar-gel test except between bovine blood and anti-buffalo serum and between human blood and anti-monkey serum. These cross reactions were considered of negligible practical importance, and the agar-gel diffusion technique was adopted in place of the ring test. While results were obtained within a few minutes in the precipitin ring-tests, it took over 24 hours at room temperature before the agar-gel tests could be read. Possibly, the slow migration of both antigen and antiserum in the agar-gel diffusion method eliminates or delays the appearance of cross-reactions.

Material tested by the agar-gel diffusion technique included 470 mosquitoes collected in a light trap located near a cattle barn at Bang Phra, Cholburi Province, where studies on mosquito-borne viruses were in progress. Results shown in Table 1 reveal that cattle appeared to be the preferred hosts for all of the species tested. Both *Aedes mediolinieatus* and *Aedes vexans* fed occasionally on chickens or on both chickens and bovines, but *vexans* showed a larger portion of mixed feedings. Although the material tested represented collections made over the period between March and September, feeding of these two mosquitoes on non-bovine hosts occurred only during April and May. (See section on arbovirus studies for further details).

Summary: Because of very broad cross-reactions obtained with certain antisera in the precipitin ring test, it was replaced by the agar-gel diffusion technique which eliminated practically all cross-reactivity. Tests of mosquitoes from the Bang Phra arbovirus study area indicated that most of those tested had fed upon cattle with occasionally blood meals being taken from chickens.

Table 1.

Source of blood meals of mosquitoes collected from light trap at Bang Phra 1966 as determined by the agar-gel diffusion technique.

Species	Total number tested	Number positive reactions	% positives with antiserum*		
			B/C	C & Ch.	Ch.
Aedes lineatopennis	130	121	100		
A. mediolineatus	62	55	96	2	2
A. vexans	158	154	66	31	3
Culex gelidus	89	88	100		
C. tritaeniorhynchus	32	32	100		

* B/C, buffalo or cow; C & Ch., both cow and chicken; Ch., chicken only

SEATO MEDICAL SESEARCH STUDY ON MYCOTIC DISEASES

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Principal Investigator:	Robert L. Taylor, LTC, MSC
Associate Investigators:	1. Burapa Chanasut, M.D. 2. Rasem Chittayasothorn, M.D. 3. George Hennard, Lt. Col. MC 4. Renoo Kotrajaras, M.D. 5. Vinitta Jotisankasa, M.D. 6. Leon J. Le Beau, Ph.D. 7. Udom Legsomboon, M.D. 8. John. H. Morris, Lt. Col. VC 9. Norman C. Negus, Ph.D. 10. Suchit Pookavej, M.D. 11. Punya Ruenwongsa, M.D.
Assistant Investigators:	1. Yupin Charoenvit, B.Sc. 2. Chiraphun Duangmani, M.D. 3. Malinee Thamrongnavasawasdi, B.Sc.

Period of Report: Annual, 1 April 1966–31 March 1967

General Information:

A Medical Mycology Section was established in October 1965 to study the mycotic diseases of Thailand. The objectives of these studies are to gain information on the prevalence and distribution of all mycoses of importance in this area of the world. Little information is currently available, therefore, collection of data to permit evaluation of each of the potentially important mycoses is essential. In addition to the cosmopolitan dermatophytes, which can be a major cause of morbidity, studies were initially concentrated on those mycoses which are most likely to be found in Thailand.

Another important facet of the activities of the Mycology Section has been to furnish training and guidance to local persons interested in medical laboratory mycology. Mr. Parimonth Khanjanarthiti, Miss Majouree Suksuwan (lecturer and instructor in Microbiology, Faculty of Chiengmai Medical School) and Dr. Vinitta Jotisankasa (Department of Medical Sciences Laboratory Service) have each spent one month in the laboratory. Training included both formal and on-the-job instruction.

Study Reports.

1. Title: Prevalence and distribution of histoplasmin hypersensitivity among Thai nationals

Principal Investigator: Robert L. Taylor, LTC, MSC

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The percentage of histoplasmin sensitivity among lifetime residents of a given area is an index of the exposure rate that can be anticipated should non-resident personnel move into the area. For this reason, Korat (Nakorn Rajasima) was selected as the first area to study. Lt Col G. Hennard, Surgeon 9th Logistical Command, assisted in the establishment of contacts resulting in the histoplasmin skin testing of 610 adult Thais (532 males; 78 females). The overall percentage of reactors was found to be 5.40 (5.82% males; 2.56% females).

The second area to be surveyed was Chiengmai province in north Thailand. Dr. Kampol Panas-Ampol, Head of the Department of Microbiology at the Chiengmai Medical School, and Dr. Leon Le Beau, University of Illinois advisor to the Department of Microbiology, made the arrangements to apply skin tests in the school system. A total of 542 (278 males; 264 females) young adults (14 to 20 years of age) were tested. The histoplasmin sensitivity was found to be 4.8 per cent with females having only a slightly lower rate than males.

The third geographic area studied was Udornthani province in the northeast of Thailand. Dr. Kasem Chittayasothorn, Director of the provincial hospital, made arrangements to apply histoplasmin skin tests in the provincial prison. The prison provided an excellent population for our purposes since it is an adult group, readily accessible for reexamination 48 hours after testing. It was also possible to select lifetime residents of given areas from the group. Subsequent studies were conducted in prisons whenever possible. Six hundred forty six adults (633 males; 13 females) were tested. The overall percentage of reactors was found to be 6.8 (6.95% males; 0.0% females).

Dr. Udom Legsomboon, microbiologist at Ubol provincial hospital, assisted in a similar survey conducted in the Ubol provincial prison. Five hundred ninety two persons (572 males; 10 females) between the ages of 16 and 70 years of age were tested. The percentage of reactors was 5.74 per cent (5.84% males; 0.0% females) and was comparable to rates found in north and northeastern areas of Thailand.

The histoplasmin sensitivity in central Thailand was determined by testing 500 male inmates (18 to 65 years of age) of Bangkwang prison (Nondhaburi, near Bangkok). Dr. Suchit Pookavej, Department of Interior, made arrangements for testing. Residents of the central plains were found to have a three and non-half fold higher reactor rate than residents of the northern provinces (18% males; females not tested).

Chanthaburi was selected as a representative area in the southeast of Thailand and 208 prisoners and 77 Thai marines were skin tested. The reactor rate among the 208 prisoners (203 males; 5 females) was 19.2 per cent, whereas among the marines the rate was 14.3 per cent. The discrepancy between reactor rates can be attributed to age distribution in the two groups. The Thai marines were all between 20 and 23 years of age while many of the prisoners were older, hence had greater opportunity to develop histoplasmin hypersensitivity.

The last geographic area studied during the period of this report was the southern peninsula of Thailand which is contiguous with Malaysia. Histoplasmin skin tests were applied to 515 inmates (482 males; 33 females) of the Songkhla provincial prison. Slightly more than 25 per cent of the males and 9 per cent of the females were reactors. The overall reactor rate of 24.3 per cent is the highest yet found in Thailand and five times greater than found in Chiengmai province in the north of Thailand.

The histoplasmin survey of Thailand is approaching completion, with information obtained from seven population centers in different geographic regions. Two additional sites in the southeast and west of Thailand may be examined in the near future.

The results are summarized in Table 1 and the histoplasmin reaction sizes (induration) plotted in figure 1. The frequency distribution of histoplasmin reaction sizes were used as an index of the specificity of these reactions among Thai nationals. The reactions can be seen to fall into a bimodal distribution with a low frequency at 4 mm. Therefore, the 5 mm criterion used in this study would appear to afford a satisfactory separation of positive from negative reactors. The plot of reactions measuring 5 mm or greater resembles a normal distribution curve with a maximum frequency a 9 mm. Similar distributions have been found in areas of endemicity, and are used to verify specific histoplasmin sensitivity and to rule out cross-reactions or non-specific reactions. The raw data reported in Table 1 must be considered in view of factors which influence the results in any histoplasmin servey; such as ratio of males to females, age, and occupation of the test population. In general, the percentage of histoplasmin sensitivity increases in the same population with every year of age. Males uniformily react in a higher percentage than do females, and occupations closely associated with agriculture or the soil have a higher sensitivity rate. In this survey age and sex are the most important differences among the populations tested in the various geographic regions. In every area except Chiengmai and possibly Korat the number of females was too low to be of significance. In Chiengmai the female rate was unusually similar to that found in males and therefore did not unduly depress the overall percentage. Adults comprised the majority of the test group in each area except in Chiengmai where the survey was conducted in the secondary school system. For this reason caution must be exercised when comparing the number of reactors in Chiengmai with other areas. A lower rate is to be anticipated among the younger people.

The results of 3690 histoplasmin skin tests in Thailand indicate a low Histoplasma capsulatum endemicity in most areas of the country with few clinical cases occurring each year. The areas of highest endemicity are the southern peninsula and the southeastern provinces of Thailand.

2. Title: Ecologic study of pathogenic fungi in Thailand

Principal Investigator: Robert L. Taylor, Lt. Col., MSC

Assistant Investigators:
1. Yupin Charoenvit, B.Sc.
2. Malinee Thamrongnavasawasdi, B.Sc.

The ecologic studies of pathogenic fungi in Thailand have centered around the study of soil, which frequently serves as the reservoir for human infections, and attempts to detect naturally acquired mycoses among the rodent population. Examination of soils for pathogenic fungi is a technique frequently used to establish the endemicity of an area or to determine a focus of infection.

Soil collections have been made from several area of Thailand including the north, norsheast, central, southeast and southern provinces. Collections were made from ecologic sites similar to those known to harbour Histoplasma capsulatum or Cryptococcus neoformans in other parts of the world. A total of 84 soil samples have been processed for recovery of pathogenic fungi using animal inoculation, direct culture, and sterilized human "hair-baiting" techniques. Mouse inoculation failed to recover H. capsulatum from any of these specimens including those collected in caves and therefore enriched with bat guano. Failure to recover H. capsulatum from soil is not unexpected and corroborates the low endemicity indicated by the histoplasmin skin test survey. Much of the soil processing for H. capsulatum was conducted concurrent with the histoplasmin survey and deemphasized when the results of both studies showed a low incidence of this organism in Thailand.

Selected soil samples were collected recently from caves on islands off the coast of southern Thailand. These caves are famous for the bird nests used in the preparation of "birds nest" soup. Arrangements were made through the Bureau of Inland Revenue to have the contractor collect swallow droppings from the floor of these caves and 12 samples were obtained from 12 different islands. As per prior agreement these samples were shipped for processing to Dr. Libero Ajello, Communicable Disease Center, Atlanta, Georgia. Results from these specimens, collected from an area in Thailand with the greatest percentage of histoplasmin reactors, are pending.

Since crytococcosis is an important systemic mycosis in Thailand, 23 soil samples contaminated with pigeon or cuckoo droppings were collected specifically for recovery of Cryptococcus neoformans. The initial recovery of C. neoformans, from natural substrates in Thailand, was accomplished by this laboratory early in 1966. C. neoformans was isolated from an abandoned pigeon nest, as well as soil containing pigeon droppings, collected in Udornthani (northeast Thailand). Additional isolations of C. neoformans have been made from similar specimens collected in Bangkok and Chanthaburi (southeast). Soils collected near Bangkok's main railroad station, and others from within one-half block from the SEATO Medical Research Laboratory yielded C. neoformans. The ecologic habitat of the three isolates from Chanthaburi differed in the fact that two were from unadulterated pigeon droppings and the third was recovered from cuckoo droppings collected from the floor of the cage.

These isolations confirm C. neoformans to be a cosmopolitan fungus and explains the reason cryptococcosis is one of the important systemic myoctic diseases in Thailand.

Fifty-eight of the eighty-four soil samples were also processed using a human hair-baiting technique for isolation of keratinophylic fungi. Microsporum gypseum was recovered from four samples and Trichophyton terrestre from thirty-seven samples using this technique. The importance of M. gypseum as an etiologic agent for diseases of the skin and hair is well known, however, the significance of T. terrestre is doubtful since it is currently throught to be a non-pathogen for humans. A previous study (Taylor, R.L., Occurrence of Microsporum gypseum in Thailand Soils. Mycologic LVIII; 1966) showed M. gypseum to be present in 39.3% of 140 soil samples collected in 70 of the 71 provinces. Recovery of M. gypseum from soil collected in 41 provinces demonstrated the wide distribution of this organism throughout Thailand. The much lower recovery rate of M. gypseum from samples collected from caves and pigeon habitats is undoubtedly a reflection of sampling from ecologic situations unfavorable for propagation of M. gypseum.

Additional Soil collections are not contemplated.

3. Title — Study of the Dermatophytes Indigenous to Thailand

Principal Investigator: Robert L. Taylor, Lt. Col., MSC

Associate Investigators:
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2. Vinitta Jotisankasa, M.D.

Assistant Investigators:
1. Yupin Charoenvit, B.Sc.
2. Malinee Thamrongnavasawasdi, B.Sc.

This study was prompted by the major medical problems [illegible] dermatophytic fungi present to the military in times of stress. The project was initiated in November 1965 in collaboration with Dr. Renoo, dermatologist at Women's Hospital in Bangkok. One morning eac[illegible] two members of this laboratory go to the clinic to collect material from patients with dermatolog[illegible] Late in July 1966, a similar collaborative effort was established with Dr. Vinitta, Laborat[illegible] epartment of Medical Science, to obtain cultures from two additional hospitals in the Bangkok [illegible] urce of material will be of value in that it will increase the number of male patients and provid[illegible] from a lower economic stratum.

Patients presenting themselves to these clinics (Women's, Tobacco Monopoly, Bangrak Hospitals) were cultured irrespective of the clinical diagnosis. Fungi were isolated from approximately 40 per cent. Cultures were prepared by first cleansing the area of the lesion with 70 per cent alcohol and transferring material (skin, hair, nail) directly to two plates of Sabouraud-Cycloheximide-Chloramphenicol medium. The plates were sealed with paper tape, to prevent contamination, and periodically examined during a 21 day incubation at 25°C. Blood agar plates were also inoculated and incubated at 37 C when clinical appearance of the lesion indicated either a possible primary or secondary bacterial infection.

The most frequently isolated organism was *Candida albicans*, 38.56%, followed by *Trichophyton rubrum*, 34.22%; *Trichophyton mentagrophytes*, 15.12% *Epidermophyton floccosum*, 6.62%; *Microsporum gypseum*, 1.89%; *Trichophyton tonsurans*, 0.94%, *Microsporum audouinii*, 0.76% and *Trichophyton concentricum*, 0.19%.

Frequency of various fungi isolated from lesions on the following areas of the body.

A. Body (trunk, face, arms & legs) (190/529) 35.92%

1.	*Trichophyton rubrum*	50.53%
2.	*Trichophyton mentagrophytes*	17.37%
3.	*Candida albicans*	13.16%
4.	*Epidermophyton floccosum*	9.47%
5.	*Microsporum gypseum*	4.74%
6.	*Microsporum canis*	3.68%
7.	*Trichophyton tonsurans*	1.05%

B. Feet (149/529) 28.17%

1.	*Candida albicans*	53.69%
2.	*Trichophyton mentagrophytes*	22.15%
3.	*Trichophyton rubrum*	19.46%
4.	*Epidermophyton floccosum*	2.68%
5.	*Microsporum audouinii*	1.34%
6.	*Microsporum canis*	0.67%

C. Groin (70/529) 13.23%

1. Candida albicans	44.28%
2. Trichophyton rubrum	35.71%
3. Epidermophyton floccosum	14.29%
4. Trichophyton mentagrophytes	5.71%

D. Nails (52/529) 9.83%

1. Candida albicans	90.38%
2. Trichophyton rubrum	9.62%

E. Hands (48/529) 9.07%

1. Trichophyton rubrum	45.83%
2. Candida albicans	33.33%
3. Trichophyton mentagrophytes	12.50%
4. Epidermophyton floccosum	4.17%
5. Microsporum audouinii	4.17%

F. Scalp (11/529) 2.08%

1. Trichophyton rubrum	36.36%
2. Trichophyton tonsurans	27.27%
3. Trichophyton mentagrophytes	18.18%
4. Microsporum canis	9.09%
5. Microsporum gypseum	9.09%

G. Oral (lips and tongue) 9/529) 1.70%

1. Candida albicans	66.67%
2. Trichophyton mentagrophytes	22.22%
3. Microsporum canis	11.11%

The incidence of fungi isolated from men (250) and women (279).

	Males	Females
Body (trunk, face, arms & legs)	30.00%	40.86%
Feet	39.20%	18.28%
Groin	15.60%	11.83%
Nails	3.20%	15.41%
Hands	8.00%	10.03%
Scalp	2.00%	2.15%
Oral	1.60%	1.79%

The results of this survey clearly indicate the importance of fungi in dermatologic lesions in the tropics. It should be stressed that the distribution of etiologic agents among this population may not be identical to those which should be expected among U.S. personnel under field conditions. Some factors influencing the results are age, sex, occupation, socio-economic level and the influence of different geographic

areas. For example, children are more susceptible to scalp infections, and infants and older people more likely to have oral disease. Women were found to have more nail infections, and men more tinea pedis. This may be due to the fact that women are more apt to have their hands immersed in water for long periods of time, whereas men in the business world are required to wear shoes for longer periods than required of women. The influence of socio-economic level, hygienic habits and local areas of endemicity are self evident.

The species and prevalence of dermatophytes encountered is comparable to those seen in many parts of the world including the United States. However, several unusual organisms were encountered. Many of the isolates of Trichophyton rubrum were unlike those seen in the United States. They were characterized by a zone of bright yellow pigment at the periphery of the colony, with the typical deep red pigment restricted to the center of the colony. Colonial and microscopic morphology of these isolates were otherwise typical. An unusual Microsporum species, with characteristics of both M. canis and M. audouinii, was isolated from gibbons in the SMRL gibbon colony. Subsequently, similar organisms were isolated from human disease. A more complete description of this organism will be included in a discussion of the dermatologic disease in gibbons. The single isolate of Trichophyton concentricum serves as a reminder that this "exotic" organism does occur in Thailand and cases of tinea imbricata could occur among U.S. personnel. The single isolate is not an indication of the prevalence of this organism since the disease is endemic to several locations in Thailand where many clinical cases occur. Bangkok is not an area of high endemicity for tinea imbricata.

Tinea versicolor, a superficial mycosis, was frequently noted among out-patients seen in the dermatology clinics, among inmates in Thai prisons and among U.S. personnel. Cases have been confirmed by microscopic examination and by isolation of a yeast organism (Pityrosporum orbicularis) thought by some workers to be the etiologic agent. Dr. Renoo has expressed an interest in this disease and a limited effort has been made to obtain serum from patients and to isolate organism from clinical lesions. Antibodies to organisms identified as P. orbicularis have been detected using an indirect fluorescent antibody procedure. Reports from U.S. medical personnel in central Thailand indicate troops in this area have a high incidence of tinea versicolor, however, the infection does not create a military problem due to the extremely superficial nature of the disease. No major effort is being expended to study this disease.

The most striking finding revealed by the analysis of cultures from dermatologic patients was the major role Candida albicans plays in the etiology of skin diseases. These findings substantiated our earlier impressions and supported the additional investigations that have been conducted with this organism. Two studies have been initiated.

The comparative merits of four media, albumin from chicken and duck eggs, and human serum for the identification of C. albicans was determined. More than 100 strains of recently isolated Candida have been studied and the conclusions are that rice agar medium with 1% tween 80 is superior to corn meal agar with tween 80, Czapek's agar and commercial chlamydospore agar. Human serum is the best of the rapid methods (2 hours vs 48 hours required for conventional media) but not as sensitive as rice with tween 80. A brief report authored by the assistant investigators is planned for publication in the Thai medical literature.

A preliminary study was also initiated to evaluate the embryonated chicken egg as a rapid, inexpensive method for determining the pathogenicity of Candida strains. Fifty three strains of Candida albicans and 17 strains of C. stellatoideae, C. krusei, C. parakrusei, C. parapsilosis, C. quilliermondi, C. pseudotropicalis and C. tropicalis were used to determine the route of inoculation yielding the most reproducible lethality in embryonated eggs. Intravenous inoculation was selected as the route for determining the relative virulence of Candida species in subsequent studies.

Relative virulence has been determined using four strains of C. albicans and one strain each of C. tropicalis, C. krusei, C. stellatoideae, C. parapsilosis and C. pseudotropicalis. All determinations were made in parallel with a standard strain of C. albicans to minimize the variation among eggs from week to week,

Seven dilutions of inoculum (standardized by viable colony counts) were inoculated intravenously into each of 10 embryonated eggs. Death of embryos was determined at 24 hours. Relative virulence determinations showed all four C. albicans strains to be similar in virulence with the other Candida strains exhibiting a much lesser virulence. A simple, practical method for separating C. albicans from the other species was sought. It was found that 24 hour cultures of Candida species washed from the slant with 5 ml. of saline, and a 1:8 dilution of this suspension inoculated I.V. into embryonated eggs would provide this separation. C. albicans produced death in approximately 50 per cent of the embryos while other Candida species were not lethal. These results indicate the embryonated chicken egg to be a less costly and more rapid substitute for the classical adult rabbit pathogenicity test. No additional studies are planned with Candida species.

In January of 1966 an unusual dermatophyte was isolated from four gibbons suffering from skin lesions. Subsequently, 72 gibbons in the SMRL colony in Bangkok were cultured irrespective of lesions, and the organism recovered from 66.7 per cent (48/72). The organism is unusual in that it is a Microsporum species exhibiting gross and microscopic characteristics as well as nutritional requirements of both M. canis and M. audouinii. The organism was referred to Dr. L. Ajello, Communicable Disease Center, Atlanta Georgia, and Dr. Irene Weitzman, Columbia University for species determination. The isolates were identified as a distinct variety but within the limits used to define M. canis. A similar organism was isolated in 1962 from a gibbon imported into Germany from Thailand, and reported in the literature as M. audouinii. Our data clearly indicate this to be a common organism among Thai gibbons and an important disease agent in the gibbon colony. The hyperkeratotic dermatologic lesions in the gibbons produced by this organism have resisted treatment and present a therapeutic problem. This aspect will be covered more thoroughly in the Laboratory Animals Section of this report. Further characterization of these isolates is in progress including the range of pathogenicity and clinical response to various therapeutic agents. Infection was established on the backs of ten guinea pigs which subsequently responded to either 10 or 20 mg/kg/day of oral griseofulvin. The clinical response with 20 mg/kg/day was better than with the lower dose, however, the gibbons have failed to respond with doses of griseofulvin as high as 40 mg/kg/day.

Experimental infection was established on the forearm of an adult male producing an inflammatory reaction (10 X 10 mm) which slowly resolved to a more characteristic "ringworm" lesion. The area remained active for more than two months as evidenced by periodic recovery of the organism from the lesion. N treatment was used and all clinical evidence of disease slowly disappeared. This organism (a dysgonic M. canis) has been isolated from four human lesions. One from a 4 year old girl (ear lesion) who owned a gibbon and again from an 18 year old girl with wrist and arm lesions. Unfortunately, the gibbon died a few days before the cause of the lesion was known and was therefore not available for culture. The 18 year old girl denied any association with animals and gibbons in particular. The other two cases were SMRL veterinarians who had ample opportunity to contract the disease from infected gibbons. The first two cases responed to the usual topical applications used for dermatophytoses, whereas the two veterinarians responded dramatically to applications of TINACTIN. The organism was shown to be sensitive (in vitro) to 10 mcg of griseofulvin. The importance of this organism in man is still questionable, but the significance in present and future gibbon colonies is apparent.

4. Title: The Occurrence of Naturally acquired Mycoses in the mammals of Thailand

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Assistant Investigators: Yupin Charoenvit, B.Sc.
Malinee Thanmrongnavaswasdi, B.Sc.

The utlization of naturally acquired infections in mammals is a techniqe commonly employed to establish the endemicity of a geographic area. Rodents and bats collected for a variety of reasons by other SMRL departments were cultured for pathogenic fungi. During the last report period, livers and spleens from 403 rodents trapped in the south of Thailand were cultured for fungi. None of these animals to date has been found to have a naturally acquired mycotic infections.

Bats collected for rabies examination were also surveyed for Histoplasma Infections. Cultures were made from (1) liver and spleen. (2) lungs and (3) intestinal contents. A total of 138 bats were cultured. Eighty bats of the genus Tadarida were collected from a cave in central Thailand and 58 bats of the genus Cynopterus were netted in Bangkok. No pathogenic fungi were recovered.

Cultures were prepared from the livers and spleens of 214 rodents collected during an epidemiological survey in the north of Thailand (Chiengmai). No pathogenic fungi were recovered.

During a recent survey of plague in the rodents of Viet Nam the lungs from 185 rats and shrews were fixed in alcohol and returned to SMRL where they will be examined for microscopic evidence of adiaspirfmycosis. Results on these specimens are pending

This aspect of the mycology program has been discontinued until areas of high endemicity can be shown using other techniques

5. Title	Support Activities
Principal Investigator:	Robert L. Taylor, Lt. Col., MSC
Assistant Investigators:	Yupin Charoenvit, B.Sc. Malinee Thamrongnavasawasdi, B.Sc.

The major support efforts were diagnostic services which were provided the U.S. Embassy Medical Unit, the 5th U.S. Field Hospital or referrals from SMRL physicians. One hundred twenty eight specimens were received and fungi recovered include: Candida albicans (24), Microsporum canis (5), Trichophyton mentagrophytes (14), Trichophyton rubrum (13). Tinea versicolor was diagnosed in 15 individuals.

The mycology section has been serving as a reference laboratory for the identification of yeasts isolated in the venereal disease studies or recovered from the intestinal tract as part of the total flora determinations in the diarrhea studies.

Histoplasmin skin tests were applied as a diagnostic aid on six referred patients.

In July three specimens in holding medium were received, from Viet Nam, for fungus culture. Pseudomonas pseudomallei was recovered from these specimens thereby confirming one of the first cases of melioidosis to occur in U.S personnel in Viet Nam.

GENERAL SUMMARY

1. Three thousand six hundred ninety lifetime residents from seven areas of Thailand (Korat-central plateau; Chiengmai-north; Udornthani and Ubol-northeast; Bangkok-central plains; Chanthaburi-southeast and Songkhla-southern peninsula) were skin tested with histoplasmin. The greatest number of reactors was found to be in the southern peninsula where the hypersensitivity rate exceeded 24 per cent. Rates in the central plains and southeast approximated 18 to 19 per cent while rates in the central plateau and the northeast areas of Thailand were between 5 and 6 per cent. These data indicate a low endemicity for Histoplasma capsulatum in most areas of Thailand.

2. Cryptococcus neoformans was isolated from pigeon and cuckoo habitats, and constitutes the first isolations from natural substrates in Thailand. Isolations have been made from materials collected in Udornthani (northeast) Bangkok (two sites) and Chanthaburi (three sites). These isolations confirm the ecologic habitat of the organism, its widespread distribution and the reason cryptococcosis is one of the most important systemic mycotic diseases in Thailand.

3. No isolations of Histoplasma capxsulatum have been made from 84 soil samples processed, using animal inoculation. Rodents and bats processed for recovery of pathogenic fungi have failed to reveal the existence of naturally acquired mycotic infections. Microsporum gypseum was isolated from soil using a hair-baiting technique.

4. Cultures obtained from approximately 2000 patients seen at the dermatology clinics of three Bangkok hospitals revealed the most frequently isolated organisms to be Candida albicans, 38.6%; Trichophyton rubrum, 34.2%; Trichophyton mentagrophytes, 15.1%; Epidermophyton floccosum, 6.6%; Microsporum canis, 1.9%; Microsporum gypseum, 1.9%; Trichophyton tonsurans, 0.9%; Microsporum audoulnii, 0.8% and Trichophyton concentricum, 0.2%. Unusual strain of Trichophyton rubrum have been isolated which differ in gross colonial appearance from those seen in the United States.

5. A variant of Microsporum canis causing dermatophytic lesions in gibbons and having characteristics of both M. canis and M. audouinii has also been isolated from human disease.

6. Antibodies in the serum of patients with tinea versicolor have been demonstrated using an indirect fluorescent antibody technique. Tinea versicolor (pityriasis versicolor is a superficial fungus infection with widespread distribution in Thailand.

7. A comparative study to determine the most reliable method for identification of Candida albicans indicates rice medium with 1% tween 80 to be the method of choice. More than 100 recently isolated strains of C. albicans were tested using four conventional media and three "rapid" techniques.

8. The pathogenicity of Candida albicans can be determined by intravenous inoculation into embryonated chicken eggs. The embryonated egg is a less costly and more rapid method than the classical adult rabbit pathogenicity test.

PUBLICATIONS

1. Occurrence of Microsporum gypseum in Thailand Soils, Robert L. Taylor, Mycologia LVIII: 648, 1966
2. Superficial Dermatomycoses at Women's Hosptial, Renoo Kotrajaras and Robert L. Taylor, Trans. Thailand Medical Assoc. 1966.
3. Occurrence of Dermatophytes amang patients at three Bangkok Hospitals, Robert L. Taylor, Renoo Kotrajaras, and Vinitta Jotisankasa. Trans. Thailand Medical Assoc. 1966.
4. Superficial Dermatomycoses in Dermatological Clinics, Bangkok, Thailand. Renoo Kotrajaras and Vinitta Jotisankasa. Dermatologia Tropica, in press.

Table I

Histoplasmin Sensitivity among Thai nationals

Location	No. tested	Males	Females	Reactors		
				Male (%)	Female (%)	Total
Korat	610	532	78	31/532(5.8)	2/78(2.6)	5.4%
Chiengmai	542	278	264	14/278(5.0)	12/264(4.5)	4.8%
Udorn	646	633	13	44/633(6.9)	0/13 (0)	6.8%
Ubol	592	582	10	34/582(5.8)	0/10 (0)	5.7%
Bangkwang (Bangkok)	500	500		90/500(18)		18.0%
Chanthaburi (Prison)	208	203	5	39/203(18.7)	1/5 (20)	19.2
Chanthaburi (Navy)	77	77		11/77(14.3)		14.3
Songkhla	515	482	33	122/482(25.3)	3/33(9.1)	24.3
Totals	3590	3287	403	385/3287(11.7)	18/403(4.5)	10.9%

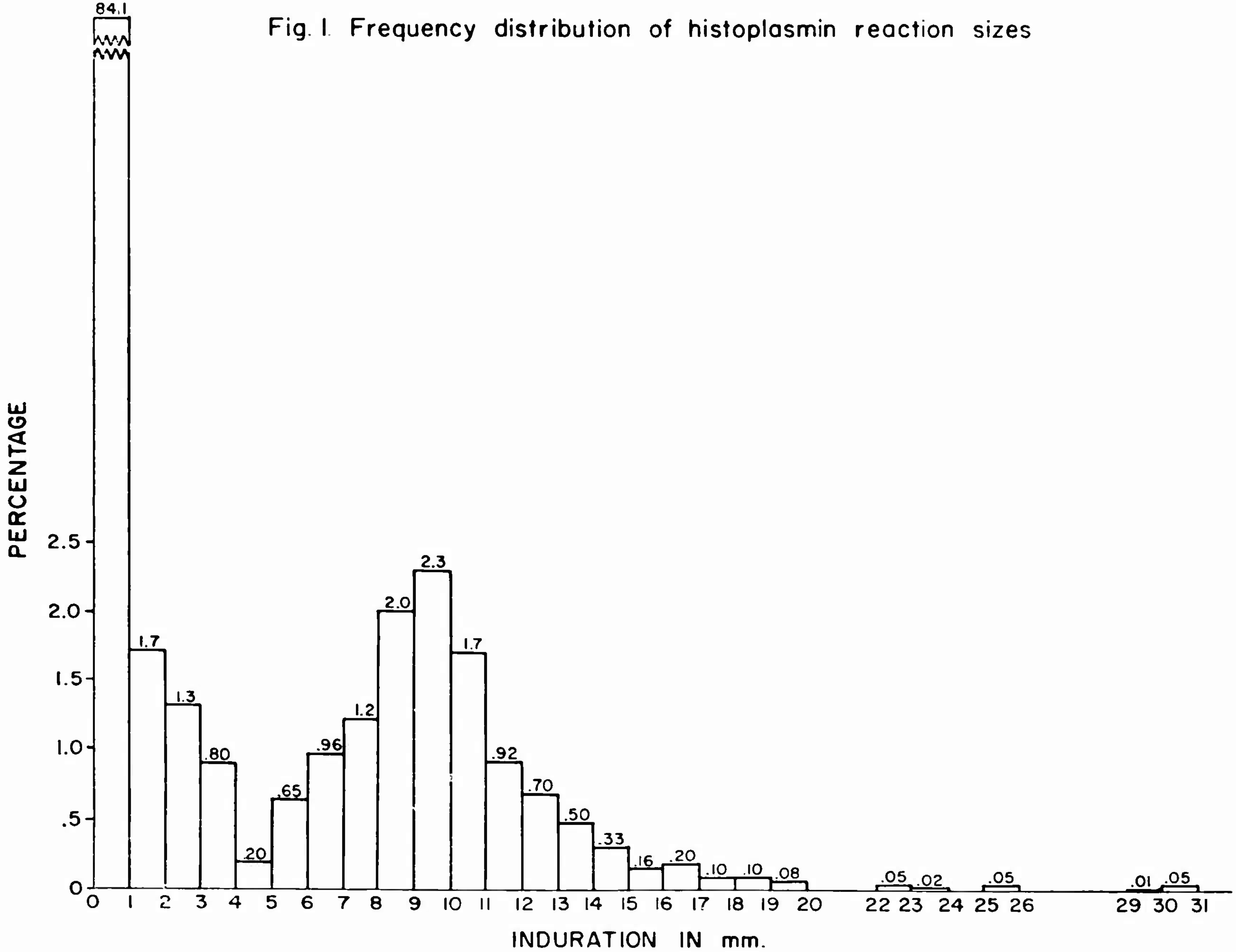

Fig. 1. Frequency distribution of histoplasmin reaction sizes

SEATO Medical Research Studies in Neurology

Coordinator:	Martin Chipman, Major, MC
Principal Investigators:	1. Martin Chipman, Major, MC 2. Udomporn Kashemsant, M.D. 3. William H. Biggers, Captain, MC
Associate Investigators:	1. Wisit Benjapongse, M.D.* 2. Philip E. Winter, Major, MC
Consultant:	Joseph F. Fazekas, M.D.

General Introduction As a result of a thirteen-week preliminary investigation of malaria and its clinical effects on the nervous system[1]. It was evident that investigations of cerebral hemodynamics and metabolism were warranted not only in malaria but in other systemic infections. The following report describes the experience and results obtained during the application of a cerebral blood flow technique.

* Physician, Phrabuddhabat Hospital, Saraburi Province.

STUDY REPORT

Title: Malaria and the Nervous System: Cerebral Hemodynamics and Metabolism in Patients with Malaria and Central Nervous System Symptoms

Part I. Cerebral Hemodynamics in Young Thai Males

Principal investigators:
1. Martin Chipman, Major, MC
2. Udomporn Kashemsant, M.D.
3. William H. Biggers, Captain, MC

Associate Investigators:
1. Philip E. Winter, Major, MC
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Reporting Period: 1 April 1966–31 March 1967

In 1948 Kety and Schmidt introduced the nitrous oxide technique for the determination of cerebral blood flow and metabolism and established normal values in fourteen healthy young men.[2] Since then a considerable amount of information has been accumulated on the effect of various drugs and disease states on cerebral hemodynamics and metabolism. During the past twenty years technological innovations, particularly the use of isotopes, have refined the determination of cerebral blood flow; nevertheless, the original Kety and Schmidt inert gas procedure and the principle on which it is based remain the standards against which other techniques are compared.

This report describes the first measurements of cerebral blood flow in Thailand, discusses the use of the Scheinberg and Stead simplification[3] of the Kety and Schmidt nitrous oxide technique, and them compares the results with those obtained by previous investigators.

Introduction. The inert gas technique is based on the Fick Principle. Applied to the measurement of cerebral blood flow, the Fick Principle postulates that the flow is equal to the ratio of the brain uptake of inert gas per unit time and the arteriovenous difference of this gas during the same period. Kety & Schmidt used 15% nitrous oxide as the physiologically inert gas and administered it to supine patients for ten minutes. During the ten minutes saturation period, multiple simultaneous superior jugular bulb and femoral artery samples were obtained, their arteriovenous differences integrated, and the resultant value placed in the denominator of the Fick equation. The numerator of the equation, representing the nitrous oxide concentration of the brain at saturation, was derived from the concentration of the inert gas in the jugular bulb at the end of ten minutes.[2]

The Fick Equation

$$* \; CBF = \frac{100\, C_{v10} \cdot S}{\int_0^{10} (C_a - C_v)dt}$$

Where CBF = Cerebral blood in ml./min./100 grams of brain

C_{v10} = The concentration of nitrous oxide in the jugular bulb at the end of ten minutes

S = The brain:blood coefficient; this factor represents the ratio of nitrous oxide solubility in blood and brain for varying hematocrits

$\int_0^{10} (C_a - C_v)\, dt$ = The arteriovenous nitrous oxide difference integrated over 10 minutes.

This technique which measures only the mean blood flow requires that the concentration of the inert gas and its rate of administration remain constant throughout the ten minute saturation period, and that arterial pCO_2 not change appreciably ($\pm$ 4.5 mm Hg)[5] during the procedure. Kety[2] and Lassen[6] have discussed the theoretical bases for the experimental assumptions which are made in this method.

Scheinberg and Stead simplified the Kety-Schmidt procedure by drastically reducing the number of nitrous oxide determinations.[3] The denominator of the Fick equation was obtained by mechanically integrating arterial and jugular bulb samples over ten minutes and the drawing a rapid jugular bulb sample whose nitrous oxide content represented the nitrous oxide content of the brain at the end of ten minutes.*

Method. Healthy males between 21 and 30 who volunteer for this study are seen by at least one of the investigators (U.K. and/or M.C. and W.B.) the day before the procedure. An explanation of the procedure is given to each patient. Pertinent history is obtained, and physical examination, chest films, urinalyses, and EKG's are then done. If no abnormalities are found the patient returns the next morning for cerebral blood flow examinations.

Before beginning the procedure each patientais placed on a stretcher cart where he rests for at least 10-15 minutes. The right mastoid tip and the left antecubital areas are then washed with Methyolate. The cutaneous and subcutaneous areas below the mastoid process and around the antecubital fossa are then infiltrated with 1% Xylocaine. A 19 gauge, 13/4 inch siliconated needle attached to a 5-ml syringe is inserted just below and anterior to the tip of the mastoid process and directed anteriorly and superiorly in the direction of the internal auditory meatus. The needle passes just beneath the base of the skull and enters the superior bulb of the internal jugular vein shortly after it exits from the jugular foramen. Figure I illustrates the course of the needle. An 18 gauge Cournand needle is placed in brachial artery. The needles are the attached through suitable adapters to the 5 stop-cock sampling manifolds which are fitted with flushing syringes and lightly glycerinated and heparinized sampling syringes. A drip containing 500ml. of 5% dextrose and water mixed with 5 mgms of Heparin keeps the jugular bulb needle patent. A dampened aneroid manometer[7] attached to the arterial manifold monitors mean arterial blood pressure (MAP). When mean arterial pressure becomes stable the manifolds are filled with blood, a 6-ml control sample is drawn from the jugular bulb, and simultaneous arterial and venous samples are drawn for pH, pCO_2 and hematocrit.

* Adapted from McHenry[4]

* The brain: blood coefficient for nitrous oxide at normal hematocrits is unity.

Immediately after re-checking the mean arterial pressure the patient begins to breath a gas mixture of 15% nitrous oxide, 25% oxygen and 60% nitrogen through a Ruebens non-rebreathing valve. As soon as gas inhalation starts the simultaneous venous and arterial blood samples are drawn at the rate of 2 ml. per minute for 10 minutes. At the end of 10 minutes the stop-cocks to the integrated samples are closed and while the patient continues to breath the gas mixture 6-mls. of venous and arterial blood are drawn in 20 seconds. The mask is then removed, the mean arterial pressure checked, and samples for pH and pCO_2 are rapidly drawn. The mechanically integrated blood samples are analyzed for nitrous oxide by the Kety modification of the Orcutt-Waters-Van Slyke procedure[7] and for oxygen and carbon dioxide contents by the Van Slyke-Neill manometric technique.[8] pH and pCO_2 are measured with the Astrup Radiometer apparatus and nomograms.[9]

Figure 2 illustrates the Scheinberg and Stead method for calculating mean cerebral blood flow (CBF), cerebral vascular resistance (CVR), and cerebral oxygen consumption ($CMRO_2$).

Results and Discussion Table I demonstrates the results obtained in 26 normal, young, male volunteers. Using the Scheinberg and Stead modification of the Kety-Schmidt nitrous oxide method we obtained a mean cerebral blood flow of 57.0 ml./min./100 grams of brain, a mean cerebral vascular resistance of 1.5 mm Hg/ml./min./100 grams of brain, and a mean cerebral oxygen consumption of 3.8 ml./min./100 grams of brain. Significant changes of pCO_2, pH and mean arterial pressure did not occur during the procedures. These results and their standard deviations are similar to those obtained by earlier investigators who used the same method (Table 2). Our results also closely approximate those obtained by Lassen and Munck.[13] (CBF 51.9ml./min./100 grams of brain with a S.D. of 8.6) and McHenry[14] (CBF 56.5ml./min./100 grams of brain with a S.D. of 7.7) both of whom used the more accurate Krypton[85] saturation and desaturation methods.

No complications occurred in the 26 patients described in this report. In an earlier group of 53 patients we had three minor complications: two vasovagal reactions which quickly responded to raising the foot of the stretcher cart and one case which developed a five minute paresis of the muscles on the right side of the face secondary to Xylocaine infiltration of the peripheral branches of the facial nerve. Fazekas has reported similar complications.[12]

Summary: The measurement of cerebral hemodynamics and oxygen metabolism in 26 young normal Thai males is described.

The bases of the Kety-Schmidt inert gas technique are discussed and the data obtained in our patients with this method are compared with those of other investigators.

Acknowledgements

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Figure I. Basal view of the skull demonstrating the method for puncturing the superior bulb of the internal jugular vein.

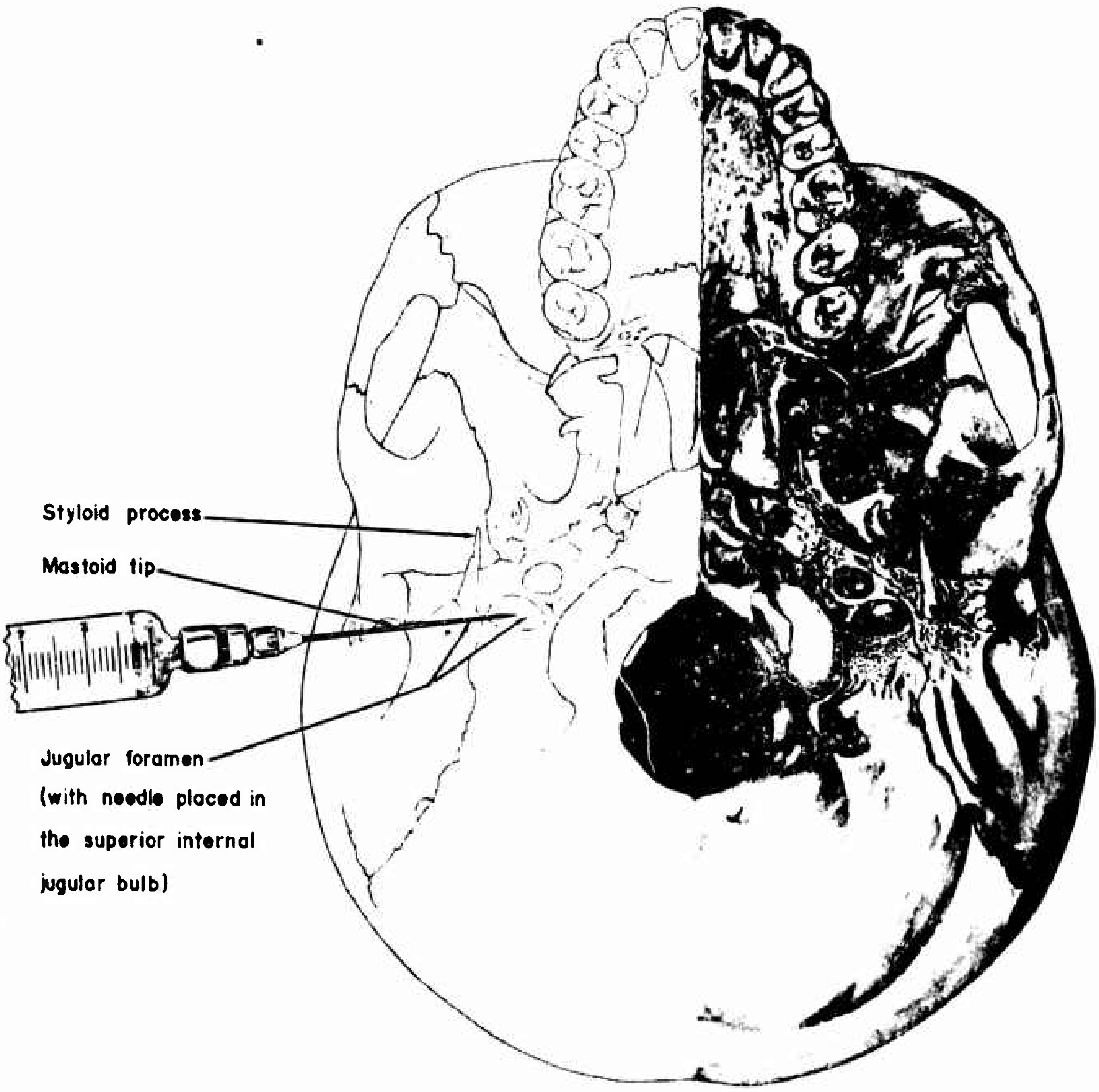


Figure 2

* An Illustration of the Scheinberg–Stead Method for Calculating Mean Cerebral Blood Flow (CBF), Cerebral Vascular Resistance (CVR) and Cerebral Oxygen Consumption ($CMRO_2$)

Subject #17

Nitrous Oxide Analyses (in Vols%)

	1st Analysis	2nd Analysis	Mean
** Control	1.18	1.18	1.18
RJB_F	5.32	5.34	5.33
LBA_F	5.31	5.34	5.33
$\int$ LBA	5.20	5 17	5.19
$\int$ RJB	4.48	4.50	4.49
$A-V_{N_2O}$			0.70

1. CBF $= \dfrac{(RJB_F - \text{Control}) \times 100}{A-V_{N_2O} \times 10} = \dfrac{(5.33 - 1.18) \times 100}{0.70 \times 10} =$ 59.3 ml/min/100 grams of brain

2. CVR $= \dfrac{\text{MAP (Mean Arterial Pressure}}{\text{CBF}} = \dfrac{84}{59.3} =$ 1.4mm Hg/ml/min/100 grams of brain

3. $CMRO_2 = \text{CBF} \times \dfrac{\text{Arteriovenous } O_2 \text{ difference}}{100} = 59.3 \; \dfrac{6.5}{100} =$ 3.9ml/min/100 grams of brain

$\int$ LBA = integrated artery sample
$\int$ RJB = integrated jugular bulb sample
$A-V_{N_2O}$ = integrated arteriovenous nitrous oxide difference

* All analyses are done in duplicate and then averaged

** RJB_F = Jugular bulb sample at saturation

LBA_F = Brachial artery sample at saturation (this sample is obtained as a control and should be no more than 0.4 Vols% more than RJB_F)

Table I

Cerebral Blood Flow, Cerebral Oxygen Consumption
Cerebral Vascular Resistance and Blood Gases in 26 Normal Young Thai Males

Subject	Age	CBF	$CMRO_2$	CVR	MAP	O_2 Content		CO_2 Content		Arteriovenous O_2 Differences	Control pH		Control pCO_2		Hct %
						BA	JB	BA	JB		BA	JB	BA mm Hg	JB mmHg	
1	29	51.3	3.5	1.9	97	17.4	10.5	49.6	54.6	6.9	7.40	7.29	41.0	52.8	38
2	22	53.9	3.8	1.8	97	15.8	9.7	48.3	55.6	7.1	7.33	7.30	49.0	50.0	39
3	25	64.2	6.3	1.1	88	17.0	9.2	50.6	58.3	7.8	7.39	7.37	41.0	45.0	36
4	24	63.6	3.8	1.5	95	19.1	13.2	47.5	52.9	5.9	7.39	7.37	41.5	60.0	44
5	22	[illegible].4	4.1	1.5	86	18.6	11.5	48.8	[illegible].7	7.[illegible]	7.34	7.28	40.0	57.0	42
6	24	55.7	4.8	0.9	90	20.3	13.1	52.5	[illegible].9	7.2	7.35	7.29	43.5	56.0	49
7	26	66.1	4.0	1.2	62	18.5	12.5	49.1	[illegible].1	6.0	7.36	7.29	40.0	53.0	44
8	30	53.0	3.2	1.6	63	14.5	10.4	[illegible]1.6	[illegible]	[illegible].1	7.33	7.31	47.0	53.5	39
9	26	49.7	3.4	1.8	89	17.7	10.9	51.6	57.4	6.8	7.35	7.30	42.0	54.0	41
10	21	71.0	4.9	1.3	90	19.9	12.6	47.9	55.0	7.0	7.40	7.33	41.0	56.2	47
11	24	62.3	4.[illegible]	1.5	91	18.0	11.3	47.4	53.7	6.7	7.39	7.32	40.5	52.0	41
12	22	48.0	2.4	0.9	90	17.2	12.1	55.0	60.0	5.1	7.39	7.32	40.0	49.0	40
13	22	59.3	3.9	1.4	84	15.9	9.4	50.5	55.7	6.5	7.39	7.32	40.0	57.0	[illegible]
14	30	45.8	2.7	1.7	80	17.3	11.4	49.9	56.2	5.9	7.40	7.36	41.5	[illegible]	[illegible]
15	26	49.4	3.5	1.7	84	19.2	12.3	49.5	55.7	7.0	7.4[illegible]	7.35	38.5	54.0	[illegible]
16	22	48.8	3.4	1.7	83	18.0	11.1	52.0	56.3	6.9	7.37	7.34	[illegible]1.0	50.0	[illegible]
17	22	65.9	3.8	1.2	76	19.8	14.0	53.7	59.4	5.8	7.15	7.40	40.5	51.6	[illegible]
18	28	47.3	3.3	1.6	76	18.2	11.2	51.6	58.7	7.0	7.36	7.33	40.5	52.5	42
19	23	44.4	3.0	2.0	87	17.2	10.[illegible]	49.9	55.3	6.7	7.32	7.27	44.0	56.0	36
20	24	71.6	3.1	1.2	86	18.5	11.[illegible]	[illegible]4.3	61.5	7.2	7.36	7.29	47.0	52.0	41
21	23	48.4	3.6	1.6	79	19.5	13.2	48.0	54.3	6.3	7.38	7.33	–	–	45
22	24	55.9	4.2	1.3	79	17.5	10.5	48.8	55.1	7.0	–	–	–	–	40
23	28	63.4	3.4	1.4	87	15.5	10.2	50.7	55.4	5.3	–	–	–	–	34
24	22	53.9	[illegible]	1.5	81	19.1	12.6	51.6	58.0	6.5	–	–	–	–	[illegible]
25	25	58.3	3.7	1.6	91	18.5	12.2	53.4	59.3	6.3	–	–	–	–	[illegible]
26	24	60.3	3.5	1.4	83	16.6	10.8	45.6	54.1	5.8	–	–	–	–	[illegible]
Mean															
	25	57.0	3.8	1.5	86	18.0	11.4	50.5	56.6	6.5	7.37	7.32	42.5	53.7	41.5
Standard Deviation															
		8.1	0.8	0.3	5.5	1.2	1.2	2.1	2.2	0.7	0.03	0.03	3.0	3.9	3.[illegible]
Number of Determinations															
		26									21	21	20	20	

Table 2

Normal Mean Blood Flows Obtained by Investigators Using the Nitrous Oxide Technique

Investigators	No. of Observations	Mean Age	CBF	S.D.	$CMRO_2$	S.D.	CVR	S.D.	MAP	S.D.	Arteriovenous O_2 Differences	S.D.
Kety & Schmidt[2]	34	25	54.0	+ 12.0	3.3	+ 0.4	1.6	+ 0.4	86	+ 7.0	6.3	+ 1.2
Scheinberg & Stead[3]	33	25	64.7	+ 12.1	3 8	+ 0.6	1.3	+ 0.2	83	+ 8.3	6.0	+ 0.8
Bernsmeier & Siemons[10]	30	37	58.3	+ 6.6	3.7	+ 0.4	1.5	+ 0.3	95	+ 11.0	6.4	+ 0.8
Fazekas et al[11]	12	32	57.5	-	3.2	-	1.7	–	94	-	–	–
Chipman et al*	26	25	57.0	+ 8.1	3.8	+ 0.8	1.5	+ 0.3	86	+ 5.5	6.5	+ 0.7

* Data presented in this report

SEATO MEDICAL RESEARCH STUDY ON NEUROPSYCHIATRY

Coordinator	Harry C. Holloway, LTC, MC Chief, Dept. of Neuropsychiatry
Principal Investigators:	Harry C. Holloway, LTC, MC David H. Marlowe, Ph.D. Richard G. Morrill, Captain, MC
Associate Investigator:	Supoch Khwanmitra, Colonel, RTA Surgeon to Supreme Command Headquarters
Period of Report:	1 April 66–31 March 67

General Information: The goal of neuropsychiatry is to study how cultural, social, and personality factors influence human behavior. Within this context, neuropsychiatric research concerns such topics as transcultural communication, advisor-counterpart relationships, adaptational problems of U.S. families abroad, and studies of socio-cultural organization of communities. There areas are being studied by a group of psychiatrists and social anthropologists. (In addition to the studies reported herein Clark Cunningham, Ph.D. of Yale University and Gertrude W. Marlowe, Ph.D., M.S. Hyg., of the University of Pennsylvania whose studies are supported by U.S. Army Medical Research and Development contracts work in close association with investigators in SEATO Medical Research Laboratory.)

The studies of transcultural communication and advisor-counterpart relationships concern factors influencing information exchange and performance in a situation where representatives of different cultures are required to collaborate in the accomplishment of a task. A study, focused on the relationship between trans-cultural communication and medical student behavior, was conducted at the Faculty of Medicine of Suan Dok Hospital, Chiengmai, where U.S. medical educators are cooperating with the Thai faculty in the development of a recently founded medical school. The results of that study are currently being analyzed. In addition, a Thai and American psychiatrist are jointly examining the relationships between U.S. advisors and Thai counterparts in the military. The influence of such factors as organizational structure, value commitments, individual adaptation, language skills, and interpretation of task definition upon the behavior of the advisor and counterpart is being evaluated.

Preliminary work preparatory to a study concerning the disturbances of thinking in organic brain syndromes has begun. This work has the goal of developing reliable clinical techniques and tests to assess the psychological manifestations of cerebral dysfunction due to neurosurgical and systemic disease. By examining how patients and their families respond to the stress of illness, information is being acquired about the incorporation of the basic symbols of Thai culture into thought patterns of individuals.

The social anthropologists associated with the Department of Neuropsychiatry are performing research into the organization of human behavior in various ethnic groups. These studies are particularly interested in the relative part played by traditional and modern health practices in the context of the community structure. Attention is being given to what people do about illness, and what their general ideas about illness are. In addition, data about kinship ties, institutional structure, religion and rituals, and economic organization of the communities are being collected in order to provide basic understanding of the ethnographic background of each area under study.

A preliminary report on a North Central Thai village has been completed and data collection in a Northern Thai village is essentially complete. Studies of Karen villages and of health practices in Chiengmai city is in progress. The study in Chiengmai city will include a study of the adaptation of U.S. families in the Thai ambient.

STUDY REPORTS

Title: Advisor/Counterpart Relationships: An Organizational Analysis

Principal Investigator:	Harry C. Holloway, LTC, MC
Associate Investigator:	Supoch Khwanmitra, Colonel, RTA Supreme Command Headquarters
Period of Report:	1 April 66–31 March 67

Objective: This study will describe the relationships between the formal and informal organization of the Army Advisory Group of MACTHAI, the mission of this unit, and the transactions between advisors and their counterparts.

Description: This study will take as its point of embarkation an analysis of the Army Advisor Group (TAAG), MACTHAI, the immediate unit to which U.S. Army advisors are assigned. TAAG is commanded by a Colonel who is directly responsible to the Commanding General of MACTHAI/JUSMAG. At present, this units is staffed by more than eighty (80) officers, of whom more than seventy-five per cent have some formally assigned advisory function. This unit and its leadership is directly responsible for giving the advisor direction, sanction, and support in his work with his counterpart.

TAAG will be analyzed as an open system; i.e., a system capable of accepting an import from its environment,[1] transforming is import into a product, and returning this product to the environment. In the process, energy is expended and work is accomplished.[2] The goal of such work is definable in terms of the unit's mission or missions. In the terms of this model, one function of unit leadership (or command) is to maintain contact with those portions of the environment that are relevant to the accomplishment of the unit's mission, (e.g., Commanding General of MACTHAI and certain units of the Royal Thai Army;) the other function is to control the internal structure of TAAG so that its various sections relate to each other and perform so that the mission of the unit is accomplished. Generally stated the unit leadership has the function of regulating the interaction between the relevant elements in the external environment and the internal structure of the unit may be regulated so the unit accomplishes its proper mission. Some of the implications of this line of reasoning may be clarified by the introduction of the concept of constraint. A constraint is any factor which modifies the choices available to a unit.

The way in which a unit accomplishes its mission is limited by internal and external constraints; e.g., the availability of personnel, the existence of an adequate technology, the financial resources available, the definition of the unit's mission. Clearly, these constraints can change. For instance, a new technology may be developed or higher command may re-define the mission. Since new constraints arise and old ones disappear, the leadership must constantly re-examine the reality of these constraints.

1. In this context, environment means everything outside of the unit boundaries of the Army Advisory Group. MACTHAI is, in this context, a portion of the TAAG's environment.

2. A.K. Rice, The Enterprise and Its Environment. Tavistock. London, 1962.

In the analysis of the Army Advisory Group, this study will collect information which will permit the formulation of the mission of the Army Advisory Group. The sub-tasks required if the unit mission is to be accomplished will be defined and the inter-relation of these sub-tasks to each other established. The inter-relationships of such sub-tasks constitutes one potential source of constraint upon unit performance. For instance, if the number of man-hours available for work is kept constant and there is an increased demand for staff reports from higher headquarters, there may be less time available for personnel to carry out advisory work, unless there is an increase in work output per man-hour. Other constraints upon the unit's activities will be identified and the reality of such constraints will be examined.

The technique which will be utilized in carrying out this phase of the study will be repeated interviews with those who have command or leadership roles within TAAG and with those individuals who fill other roles within the organization. These interviews will aim at collecting information about the work these individuals perform and its relevance to the unit mission. Data from this source will be formulation of the functional relationships within the organization, these formulations will be presented in form of working papers to appropriate representatives of the unit for critique. Perhaps the most important function of these working papers will be to elicit further information about the unit organization.

The purpose of the unit analysis is to establish a clear picture of the work group of the advisor. It is anticipated that this will provide information about: the formal aspects of the definition of the advisor's job, the rewards he receives, the constraints upon him, and his contribution to the unit mission. In this context, the advisors and the unit's formulation of the advisory role will be examined.

The study of advisor-counterpart relationship extends this project beyond the boundaries of the unit and beyond cultural boundaries into the area of cross-cultural research. Most simply and altruistically stated, the goal of the advisor and the counterpart is to enter into a cooperative relationship in which the advisor renders advice and assistance to his counterpart which will be used to increase the efficiency of the Royal Thai Army. Even if this goal is accepted by both participants, the task which faces the advisor and his counterpart is still formidable.

The advisor and his counterpart were reared speaking different languages, and they have learned to place different values on certain classes of behavior (e.g., respect for authority.) Their concepts of what constitutes cooperation versus obsequiousness, advice versus criticism, and modern versus non-modern may be quite different. Their usual techniques for evaluating their own personal impact upon others may be invalid in their transactions with each other since such techniques are frequently based upon implicit, culturally-shaped assumptions about the meaning of a given bit of behavior; the difficulty of interpreting a smile is a classic example of this problem. In a more general sense, the styles of information collection and evaluation may be quite different for the advisor and his counterpart.

In addition, the behaviors of the advisor and the counterpart are influenced by their individual needs, such as their need for social advancement, sensual gratification, friends, and familiar surroundings. The way in which an individual chooses to gratify his needs as well as his response to what he perceives as his counterpart's needs may be important in determining the character of the advisor-counterpart transaction.

In order to describe the behavior relevant to these issues, one of the investigators will act as a participant observer at selected field Advisory Detachments of TAAG. Advisors and counterparts will also be interviewed, using a semi-structured interview format. These interviews will be conducted by the principal investigator (a U.S. psychiatrist) and the associate investigator (a Thai psychiatrist.) Data concerning the advisors' and counterparts' perception of each other and their perception of their accomplishments vis-a-vis their relationship will be investigated. Personal data about advisors and counterparts will be held in strictest confidence.

Data collected will be recorded in field notes and on magnetic tape. These data will be used to describe the organizational structure of TAAG and the relevance of the structure to the performance of the advisor's task, and to assess the significance of the transcultural transactions between advisor and counterpart

as a potential constraint on the performance of TAAG's mission. The collection of raw data will be carried out between 1 January 1966 and 1 July 1967. Further reduction of data and write-up will be accomplished between 1 July 1967 and 1 January 1968.

Progress: Individuals assigned to various duties within the Army Advisory Group have been interviewed concerning their conceptualization of their assigned duties and their activities related to their carrying out of these duties. Similar interviews have been obtained by a more limited number of Thai counterparts. Such information has been obtained from officers working with the staff officers in Bangkok and field advisors. Interview data have been supplemented by information obtained from participant observation of advisors in the work situation. The data accumulated to date are too limited to permit the investigator to draw any firm conclusions at this time. However, from inspection of the data, it does seem that certain concepts like "the oriental mind," and "losing face" are frequently used among the advisors to explain the behavior of Thai counterparts and to explain the advisor's behavior vis-a-vis his counterpart. The conceptual content of these and other common place stereo-typic explanations of behavior will also be investigated in some detail.

The initial design has been modified. It had been anticipated that work on this project would be completed by 1 July 1967. The target date for completing data collection is now 31 December 1967. During the next year individual interviewing of advisors and counterparts will be emphasized. More information will sought about how they establish reliable interpersonal relationships, exchange information and resolve conflicts. The amont of participant observation in the field will be less than originally planned.

Summary. During the past year data has been collected by interviews and participant observations. In the immediate future the study will emphasize the use of individual interviews to obtain information about interpersonal behavior in the transcultural setting.

STUDY REPORT

Title: Cross Cultural Communication: The Interaction of the American Medical Educator with His Thai Colleagues and Students

Principal Investigator: Richard G. Morrill, Captain, MC.

Period of Report: 1 April 1965 – 31 March 1966

Objective: To observe and describe interactions between United States medical educator advisors, Thai medical faculty, and Thai medical students, and attempt to identify and relate major variables influencing cross cultural communications in advisor counterpart and teacher-student role relationships.

Description: At the invitation of the Thai Government with the sponsorship of the University of Illinois School of Medicine, and with financial support of USOM, a group of American medical educators have assumed the dual roles of advisors to the Thai faculty and teachers at the Chiengmai Medical School, Chiengmai, Thailand. This situation provides an opportunity to collect concurrent data from the participants in a cross cultural relationship.

This study is investigating cross cultural communications in this setting. Particular attention is being paid to the two main role relationships in which Americans and Thai participate, advisor-counterpart and teacher-student.

With regard to the advisor-counterpart relationship the dynamics of the interpersonal communication across a cultural boundary will be described. The demands and behaviors associated with the advisor role will be emphasized. The investigator is attending individual relationships and the social dynamics of group situations where American and Thai faculty interact.

American teacher-Thai student communication patterns has been studied by observing behavior in small groups. The group involved in teaching rounds have been the group most intensely observed. The investigator has routinely accompanied certain of these groups and collected data concerning verbal interaction in English which can be recorded on a verbal behavior rating scale. These same students have also been observed by the Thai research assistant in teaching rounds conducted in their own language by the Thai faculty for comparative study of their behavior.

The data derived from the naturalistic observation of advisor-counterpart and teacher-student interactions has been supplemented by information derived from structured and unstructured individual interviews with the participants. Simultaneous translation by a translator has been employed when appropriate.

In the course of this study the Thai students have received tests to evaluate their language proficiency and additional questionnaires to assess such factors as ethnic affiliation and to record significant demographic data (age, ethnic background, area of origin etc.). One goal is to correlate the variable of Thai student verbal behavior occurring on teaching rounds involving participation of U.S. teachers, with language ability, grades, ethnic affiliation (genetic and attitudinal), and area of origin within Thailand.

Progress: The training of research assistants in techniques of rating group interaction was completed. A number of medical and surgical rounds involving Thai and U.S. teachers and Thai students were rated on a number parameters of verbal interaction. The students participating in these rounds were tested for their English speaking ability and their comprehension of English. Their responses to the U.S. advisor-teachers were evaluated using the Thai Osgood Semantic Differential. The demographic data, social background and scholastic performance of all students participating was obtained. Selected students were interviewed for detailed life histories as well as factors related to future planning and their responses to their experiences with U.S. medical educators.

Members of the Thai faculty and the University of Illinois party were interviewed to sample factors relevant to their interaction with each other and with the students. In addition, the principal investigator had an opportunity to act as an instructor teaching the use of behavioral principles in evaluating pharmacological principles. This impressionistic data obtained by observing the interaction of faculty, U.S. advisors and students in structured and unstructured situations will be compared with the reports of advisors and their counterparts of their interactions.

Data collection in this project was completed 10 December 1966 and the assembled data will be annlyzed over the next six months. The principal investigator returned PCS to WRAIR, Washington, D.C., on 15 December 1966.

Title: Medical Beliefs and Behavior in Culture, Social Structure, Internal and External Group Relationships in North Thailand

Principal Investigator: David H. Marlowe, Ph.D.

Objective: This study focuses upon the role played by medical beliefs and behavior within the social system and in the internal and external relationships of an upland minority group of Northern Thailand. Its purpose is to analyze and compare those aspects of social behavior organized in terms of concepts of health, illness, curing and "preventive medicine" with those organized in terms of kin and other social relationships, economic production and exchange, religion and ritual, and other aspects of social relationships. It examines these relationships in terms of family, village, and region; contrasting variants within the same cultural group, S'kaw Karen, and analyzes the relationships of specific villages and areas of S'kaw Karen with members of other ethnic groups, primarily North Thai and Meo.

Description: The present work is being carried out in two primary research areas: Tambol Borkeo, Amphur Samerng, Changwad Chiengmai; and Tambol Mae Klang, Amphur Chom Tong, Changwad Chiengmai. The first research center is at the S'kaw Karen village of Norn Klissu Tambol Borkeo, a hamlet of sixteen households and eighteen families. There are two secondary hamlets which arose from Klissu, which are also included in the primary research focus, Klissu Ki and Klissu Ta. The second research site is the lowland S'kaw village of Mae Tia Glo, Mae Klang, Chom Tong. This latter village is comprised of twenty-one households with twenty-six nuclear families. Also included in the primary study is a secondary hamlet, Moh Ti Koh, of thirteen households which took its origin from Mae Tia Glo some forty years ago.

In both of these primary centers the standard techniques of social anthropological field research are employed; participant observation, depth interviewing, photo-recording, tape recording of verbal materials, and extended questionnaire protocols. A family by family base of detailed data covering all pertinent areas of study is being built up. In addition to the primary research areas, a more selected data base on other Karen communities is being built up through questionnaires, surveys, and distributional studies in hill villages of Central, Central Western, and South Central Chiengmai Province.

General Description: The Karen are the largest upland minority group in Northern Thailand. Present unofficial census figures seem to indicate a total population in excess of 125,000, some 45% of all hill people in the North. Of these some 80% are estimated to be S'kaw; i.e., that group of Karen who call themselves Bukunyo or Bukunyo Jraaw. For the most part they live in small settlements of an average size of 14-17 house holds in the high valleys and [illegible] ridges of Chiengmai, Mae Hongsorn and Tak. There are as well a large number of lowland villages at the base of a number of foothill areas. There would appear to number about twenty or so households on the average.

The pattern of wider socio-political integration of the Karen village is shifting and variable; different areas of life and types of social events call forth different wider group relationships. The fundamental [illegible] the individual hamlet, and it may be taken as given that this comprises the maximally corporately [illegible]ed group for 80-95% (dependent upon geographic area) of all normal activities. Each hamlet has its [illegible] ritual leader or Geh Sapwaa Damukha, and its own secular leader, Geh Sapwaa-the latter is either the government appointed Payaiban (i.e., village headman) or his deputy. In many cases these roles are filled by the same person. In all affairs involving the government the individual hamlet functions as a part of the government Mu (i.e., village) under the Puyaiban's responsible in turn to the Kamnan (chief commune official) and decisions are taken by consensus of the adult males in all the hamlets of the Mu.

Certain events, such as weddings and funerals, require cooperate participation by a wider group of hamlets, usually all of those hamlets which have split from a single village within the past forty or so years. Normally these hamlets are no more than fifteen minutes walk away from each other. Unlike more recently

migrant hill people, the Karen village is geographically stable and most new villages are founded within or near the valley of traditional habitation. This area of Vwaw represents the widest corporate group and is defined primarily in terms of land usage rights. This is the group that traditionally has the sole right to cultivate upland fields in a given area. For the most part, the people of any of these given areas are a self acknowledged complex of bilateral consanguineal and affinal kin. More pertinent ties are those established both between complexes of hamlets in a given area and other "areas" by "husband exchange;" i.e., Karen villages are ideally preferentially exogamous with a young man marrying and taking up residence in the wife's village. These "exchanges of husbands" appear to follow definite reciprocal patterns.

Economically, most Karen are subsistence farmers with a minority of larger proprietors who are rice exporters, and another minority of landless people who engage in a variety of forms of wage labor; wood-cutting, elephant handling, mining, agricultural labor, etc. Preferentially the agricultural system in Borkeo, Chom Tong, and Mae Chaem is based on wet rice culture with secondary reliance on shifting cultivation of upland rice. In Borkeo and the high upland areas of Mae Chaem there has been a marked shift in recent years to the cultivation of the opium poppy as a cash crop following its introduction by the Meo, compounded in Borkeo by the loss of much paddy land to the tin mines. Stock raising, gathering of forest products, and cultivation of secondary crops; e.g., peanuts, garlic, onions, and soy beans all represent ancillary sources of income.

It is also important to note that the Karen, like all other hill people in the north, do not exist in either social or economic isolation but are tied to their neighbors, both North Thai and other hill people, in an intricate web of social and economic relationship.

Progress: Good progress has been made during the past year. A second research center, Mae Tia Glo at Chom Tong was chosen, a field house built and work begun in July of 1966. The ensuing months have seen the amassing of a great deal of data, both specific to the ethnography of Mae Tia Glo and comparative materials relative to both variance from and similarity to the Borkeo research area. Surveys centering on the use of the traditional herbal pharmacopia, use of government medical facilities, and the relationships of types of healing and curing ceremonies to cult and self-identification differences among S'kaw Karen groups were carried out in Mae Sarieng (May 1966) and the Doi Intanon area of Chom Tong (June 1966). A further extensive survey expected to ultimately reach 700-1000 families in Amphurs Mae Chaem, Sanpatong, and Chom Tong (Changwad Chiengmai) is presently under way. This latter is a general socio-economic and medical resource utilization questionnaire, that will also serve to delineate the gross inner and outer relationships of the Karen natural social areas (Vwaw) of Central, West Central and South Central Chiengmai.

While limitations of space preclude any extensive discussion of substantive materials gathere during the past year, the following brief discussion of the Karen descriptive and classificatory system for illness will give an idea of the direction of the past year's work.

The initial descriptive/classificatory differentiation that Karen make of illness is the dichotomy: whole body illness versus part body or localized symptom illness. This initial description is a linguistically founded mode of classifying and describing. The category of whole body illness involves fever and/or feeling states and, syntactically, represents the only area of conceptualization of illness in which the self is the object acted upon by the "illness entity." Injury and assault are handled equivalently in Karen syntactic structure.

These three cases are:

Fever		Tukabuu naa o Fever you (obj) has
	or	Fever has you

and three generalized feeling states:

Feeling not good	Tadaamuu naa o Feel no good you (obj) has
or	Feeling not good has you
Feeling sick	Tadaagi naa o Sick you (obj) has
or	Sick feeling has you
Feeling very sick	Daachaa naa o Sick (or hurt) you (obj) has
or	A very sick (or hurt) feeling has you

All other illness is linguistically handled as possessed by the individual afflicted. There are two gross conceptual categories:

1. A known disease entity: these are either classifiable as generalized "visible symptom" ailments, measles, pox diseases, skin eruptions, cough; e.g.,

Nuh daa chaa po paw o	You (subj) sickness redspots have You have measles

or a collection of associated symptoms commonly enough occurring to be considered a single disease entity:

Nuh badaamu o You "cold" have	You have a cold
"cold"	light fever, running nose, sore throat, cough, etc.

2. The most extensive category used by Karen in describing and classifying illness. Name of body parts and associated state seen as possessed by the individual afflicted: e.g.,

Nun huh puu chaa o You stomach hurt have	You have a stomach ache

Most illness is presented in terms of this final category, that is; as a list of body parts and associated state. Thus, the normal response to the question:

Daachaa naa a ? Sick you has ?	What sickness <u>has</u> you ?

Will be a body part + state list

Yuu huh puu chaa o	I have a stomach ache
Cosa chaa o	headache
Huupuuluu o	diarrhea

The most common associated states in order of occurrence as symptom presentation are:

1. Chaa — Hurt or ache. This is the most usually presented.
2. Krrey — Hot, hot and upset; as huhpuu krrey, stomach hot and upset.
3. Kuh — Heavy feeling.
4. Ssuu — Irritated and aching; used of eyes only.
5. Klee — Cold, chill.
6. Daagi — Sick feeling, sometimes used as a localized qualifier in addition to its use as a whole body state possessing the individual.

None of these conceptual categories is seen as mutually exclusive. An individual may respond to the question: "How are you sick?" "A fever has me, I have a cold, hot stomach, etc." dependent upon subjective importance of individual symptoms to the individual at the moment of presentation.

All illnesses either as entities or collections of body part states are both classified as either large illnesses or small illnesses, and are also perceived as having normal temporal spans and intensities.

Small illnesses, or daachaa daa do daa chii, are those that are at worst an inconvenience, are non incapacitating, and do not prevent the individual from performing the bulk of his or her daily chores. A large illness or daachaa luu anah guu duu, is one that does incapacitate ot prevent an individual from performing his daily chores.

The normal span and intensity of an illness might best be defined as the period of anticipated and acceptable indisposition, pain, or dislocation of normal routine. During this period little thought is given to anything but symptomatic relief. The usual medications that will be used, if medication is resorted to at all are aspirin, APC, and traditional herbals. "Small illnesses" are usually not medicated at all and many people consider the concept, "goes away without using medicine" to be a necessary attribute of small illnesses. If causative agents are thought of at all they are thought of in generic terms, and are those things that are part of the ordinary background of living, heat, cold, sun, wind, food, etc.

As further data is collected, reduced, and analyzed the goal will be to establish the relation between the conceptual classes of illness present above, other conceptual classes, health oriented behavioral patterns, and the other social relationships observed in the Karen populations being studied. The findings in one community of Karen will be compared and contrasted to the forms found in another community.

Summary: The study of medical beliefs and behavior in culture, social structure, internal and external group relationships in North Thailand continues as per the research program. Comparative data collection in all sub-field stations will continue throughout the year in both research centers, Samerng and Chom Tong. It is anticipated that the coming year will see the completion of gross data gathering and an intensification of detailed data gathering and analysis.

SEATO Clinical Research Study on Nutrition and Metabolism

Coordinator:	Aree Valyasevi, M.D. Chief, Thai Component Clinical Research Center
Principal Investigators:	Aree Valyasevi, M.D. Vichai Tanphaichit, M.D.
Assistant Investigator:	Pranee Phongbetchara, B.Sc.

Period of Report:	1 April 1966 – 31 March 1967

GENERAL INFORMATION

The project "Clinical and Biochemical aspects of Beri-beri" has been under study during the period covered in this report. The study has been conducted on patients admitted to the Siriraj Hospital and presenting the clinical manifestation of Beri-beri.

STUDY REPORT

1. Title: "Clinical and Biochemical Studies of Beri-beri in Infants and Adult"*

Principal Investigators: Aree Valyasevi, M.D.
Vichai Tanphaichit, M.D.

Assistant Investigator: Pranee Phongbetchara, B.Sc.

Objectives

To correlate clinical manifestations of beri-beri with the level of urinary thiamine and erythrocyte transketolase before and after treatment with Vitamin B. Normal levels of urine thiamine and erythrocyte transketolase in Thai subjects will also be determined.

Description

Patients who are suspected of having beri-beri, are selected for study. Approximately 10 to 20 normal subjects will also be studied in the same manner and will serve as controls.

Routine history and physical examination were performed and recorded on all subjects. Dietary history was taken in detail, and signs and symptoms of nutrition deficiency diseases were carefully evaluated, according to the ICNND Manual for Nutrition Surveys[1]. Laboratory examinations included routine urine and blood, chest x-ray, electro-cardiogram and were also obtained.

Twenty-four hour urine samples were collected on admission and after the thiamine administration. The samples were preserved by acidification with Hcl. Determination of urine thiamine was accomplished by thiochrome method, as described in the Manual for Nutrition Surveys[1]. Urine creatinine was determined using auto analyzer.

Blood specimens were obtained on admission and periodically after thiamine administration. Erythrocyte transketolase activity was measured by the method of Brin[2]

Progress

Thus far, only three patients have been studied. All of them came to the Siriraj Hospital because of pitting edema, fatigue on slight exertion and parasthesias. The results of Erythrocyte transketolase activity demonstrated elevated transketolase 76, 85, and 40 percent on admission to the hospital; then decreased to 5, 0, and 1 percent respectively one hour after administration of 100 mg thiamine intramuscularly. Clinical improvements, including weight loss etc. were also noted after the therapy. Subsequent ETK activity studies disclosed lower than normal values (20% stimulation) according to ICNND[1]. standards.

The urine thiamine determination are in progress. No conclusions can be drawn at present time.

* Support in part by USPH Grant NIH A 5921 given to Dr. Paul Gyorgy

SEATO MEDICAL RESEARCH STUDY ON RABIES

Coordinator: Paul C. Smith, CPT, VC, Asst Chief of Veterinary Medicine

Principal Investigators:	Paul C. Smith, CPT, VC Richard O. Spertzel, MAJ, VC
Assistant Investigators:	Kwanyuen Lawhaswasdi, D.V.M. Jack S. Stanton, MAJ, VC William E. Vick, CPT, VC
Period of Report:	1 April 1966-31 March 1967

GENERAL INFORMATION:

Previous studies indicating the ubiquity, and high incidence of rabies in the canine population of Thailand, tend to be confirmed by the number of positive cases among specimens submitted for diagnosis to this facility. Surveys of several species of rodents and other small mammals have revealed that a significant number of them are harboring rabies infections. Studies are being conducted to determine if these animals exhibit classical symptoms of the disease or if they are, in fact, asymptomatic carriers. Bat rabies has been diagnosed in frugivorous bats in Thailand. Previously, Southeast Asia had been considered as an area free of bat rabies. Recently a survey of the stray dog population of Bangkok has been instituted.

STUDY REPORTS

1. Title: Rabies Diagnostic Service

Principal Investigators:	Paul C. Smith, CPT, VC Richard O. Spertzel, MAJ, VC
Assistant Investigators:	Kwanyuen Lawhaswasdi, D.V.M. Jack S. Stanton, MAJ, VC William E. Vick, CPT, VC

Objective: To maintain a facility that will provide a rapid and accurate rabies diagnostic service in potential human exposure from animal bite cases.

Description: The use of the Fluorescent Antibody Technique, Seller's Stain and animal inoculation tests provide both accurate and timely information.

Progress: The results of suspect specimens submitted during this report period are shown in table I.

Table I

Species	#Examined	#Negative	#Positive
Dog	142	76	66
Cats (Domestic)	26	20	6
Human	1	0	1
Horses	2	0	2
Ox	1	0	1
Cats (Sylvatic)	4	2	2
Others	32	32	0
Totals	208	130	78

There were a total of 78 positive cases among the 208 suspect specimen submitted. More than 37% (37.5%) of the specimens were positive by the fluorescent antibody test and the mouse inoculation tests.

STUDY REPORTS

2. Title: The prevalence of sylvatic rabies in Thailand.

Principal Investigators: Paul C. Smith, CPT, VC
Kwanyuen Lawhaswasdi, D.V.M.

Assistant Investigators: Jack S. Stanton, MAJ, VC
William E. Vick, CPT, VC

Objective: The objective of this study is to determine the prevalence of sylvatic rabies in a number of potential reservoir hosts.

Description: Rodents, bats and other small mammals were randomly trapped and speciated by a field team from the Department of Parasitology, SMRL. The heads, frozen in dry ice, were shipped to the laboratory. Fluorescent antibody tests were conducted on the brains and positive results were confirmed by intracranial inoculation of weanling white mice. Dog heads were picked up at the Stray Dog Apprehension Center in Bangkok, and their brains and salivary glands examined for evidence of rabies virus by the previously described metho...

Progress: The results of surveys are given in table I. Attempts are being made to determine if the rodents involved in the survey are harboring inapparent infections or if they exhibit classical symptoms. Ten immature Bandicoot rats were inoculated intramuscularly in the quadriceps region and subcutaneously in the neck region with 0.3 ml. per site of a 20% mouse brain suspension. The suspension represented a composite of all the bandicoot isolates. Pertinent data from this experiment is shown in table II.

Seventy-nine dog-faced fruit bats, Cynopterus brachyotis, were trapped in Kanchanaburi, Thailand. These were examined by the fluorescent antibody technique and two were shown to be positive for rabies. The results were confirmed by mouse inoculation and serum neutralization. The isolate had the peculiar properties of a long incubation period (18 days) and unusual clinical symptoms. The clinical syndrome was characterized by a creeping paralysis of 4-5 days duration. The study is continuing in other areas and in different species.

A survey of the Bangkok stray dog population was begun in an attempt to confirm previous findings. Progress so far in the examination of 50 specimen taken from the municipal impounding facilities has revealed 1 active case of rabies.

Pilot studies on therapeutic regimen of infected mice and follow-up of definite exposures in Thai nationals are planned in conjunction with the departments of Neuropsychiatry and Epidemiology.

Summary: Rodents, bats, and stray dogs in Thailand have been shown to be infected with rabies. These animal populations are harboring active infections and must be considered as reservoir host in any eradication campaign to be undertaken by the government. The possibility of rodents harboring inapparent infections cannot be eliminated. The surveys are continuing.

Table I

Rodent and Small Mammal Survey for Prevalence of Rabies

Species	# Examined	# Positive	% Positive
B.*bengalensis	375	7	1.87
R.*norwegicus	192	9	4.69
R. berdemorei	2	0	0.0
R. exulans	236	6	2.54
R. rajah	16	1	6.25
R. rattus	58	2	3.45
Mongoose	2	0	0.0
S.*murinus	30	2	6.67
B. indicus	126	10	7.94
C. brachyotis**	79	2	2.5
Totals	1,037	37	3.53

* R..Bandicoota, R..Rattus, S..Suncus (Insectivore-common houseshrew)

** Cynopterus Brachyotis (Dog-faced fruit bat).

Table II

Rabies in the Immature Bandicoot Rat

Rat #	Symptoms	Death/Days P.I.	Duration of Illness/Days	FAT Results	MI Results
1	Lethargy, Paralysis	14	2	Positive	Positive
2	Lethargy, Paralysis	16	5	Positive	Positive
3	None noted	19	0	Positive	Positive
4	Lethargy, Paralysis	21	2	Positive	Positive
5	Ruffled hair, Lethargy, Paralysis	33	3	Positive	Positive
6	None noted	(79)*	0*	Positive	Positive
7	None noted	(113)*	0*	Negative	Negative
8	None noted	–	–	–	–
9	None noted	–	–	–	–
10	None noted	–	–	–	–

* Animal sacrificed.

SEATO MEDICAL RESEARCH STUDY ON RENAL STUDIES

Coordinator:	John P. Malloy, Major, MC
Principal Investigators:	John P. Malloy, Major, MC Channivat Kashemsant, M.D.
Associate Investigators:§	Pensri Makaranond, M.D. Rampai Sribhibhadh, M.D. Suchartic Indraprasit, M.D. John T. Lebow, SSG Sangchan Satrarasook, M.D.
Period of Report:	1 April 1966 - 31 March 1967

STUDY REPORTS

1. Title:	Fluid Compartmentalization Studies in Thai Subjects
Principal Investigator.	John P. Malloy, Major, MC
Associate Investigators:	1. Pensri Makaranond, M.D. 2. Rampai Sribhibhadh, M.D. 3. Suchartic Indraprasit, M.D. 4. John T. Lebow, SSG
Period of Report:	1 November 1966-31 March 1967

Objective-There are firm data in the literature for normal values for various fluid compartments including total body water, extracellular fluid, intracellular fluid, and blood volumes. All these studies have been performed on American or European subjects. This present study is undertaken to measure fluid compartments in normal Thai subjects to determine if genetic, nutritional and enviromental factors cause significant differences from accepted western standards.

Method of Study-Asymptomatic individuals who volunteered for the study were initially screened for organic disease by the following complete history and physical examination, complete blood count, serology, stools for ova and parasites, and a chest x-ray. If none of the above revealed significant disease the patients were then admitted to a metabolic ward and subsequently studied in a post-absorptive state in the following manner. An indwelling venous catheter was inserted into a peripheral vein and was used to inject the following isotope: At time zero 250 micro curies of triated water: Blood specimens were drawn at 120, 180, and 240 minutes. These were processed according to the method of Werblin (1). Fifty (50) micro curies of NA S04 (S35) were then injected and blood specimens drawn at 20, 40, 50, 60 minutes and subsequently processed according to the method of Walzer et. al (2). Both the tritium specimens and the S35 specimens were then counted in a liquid scintillation counter and using standard dilution formula total body water and extra celluiar fluid were calculated.

Red cell mass and plasma volumes were then determined according to the method of Albert (3).

Results-A total of 193 subjects have been studied to date in various age groups as shown in table 1.

To date the data have been completed and analyzed in only the initial 22 subjects. These were all young adult males and the data for this group are presented in table 2.
Figure 1. Represents the correlation between the total body water and body weight. There is good correlation for T.B.W. and body weight although the regression coefficients have not yet been determined. Figure 2. Represents the correlation between extracellular water and body weight and figure 3 the correlation between intracellular water and body weight. There is good correlation between total body weight and intracellular fluid but to date correlation coefficients and regressions have not been completed.

Discussion-The use of triated water is the simplest and most reproducable method available to determine total body water. The mean value for this group is not significantly different from western standards compiled by Moore (4). In this relatively small group that has been analyzed, there were two grossly obese subjects which may be masking a slight increase in total body water in the remaining patients. This is suggested by the larger standard deviation for body weight than for the total body water determinations. Analysis of the remaining patients in this age group will be necessary to confirm this impression.

The values for extracellular fluid are slightly lower than western standards. Intracelluler water is higher than the expected values taken from Moore's (4) series. Since intracellular water is the best parameter of lean body mass these higher values may reflect a better degree of physical conditioning for this group of subjects. Poor nutritional status would not account for this apparent increase n intracellular fluid and none of the subjects appeared malnourished clinically. Subsequent analysis of the data in the other patients that have been studied, should elucidate differences due to sex. Most interesting will be the differences produced by aging.

The values for red cell mass are below western standards while values for the plasma volume are in the expected ranges.

References

1. H. Werblin, I Chikoff M.R. Imada. Proc. Soc. Exp. Biol and Med. 102, 8, 1959

2. Walzer, M., Seldin D.W. and Grollan, A

3. Albert S.N., Blood Volume, Thomas, Springfield, Illinois 1963

4. Moore, F.D. Body Cell Mass, Saunders, Philadelphia 1963

Table 1

Total of 193 subjects studied to date in various age groups in each sex

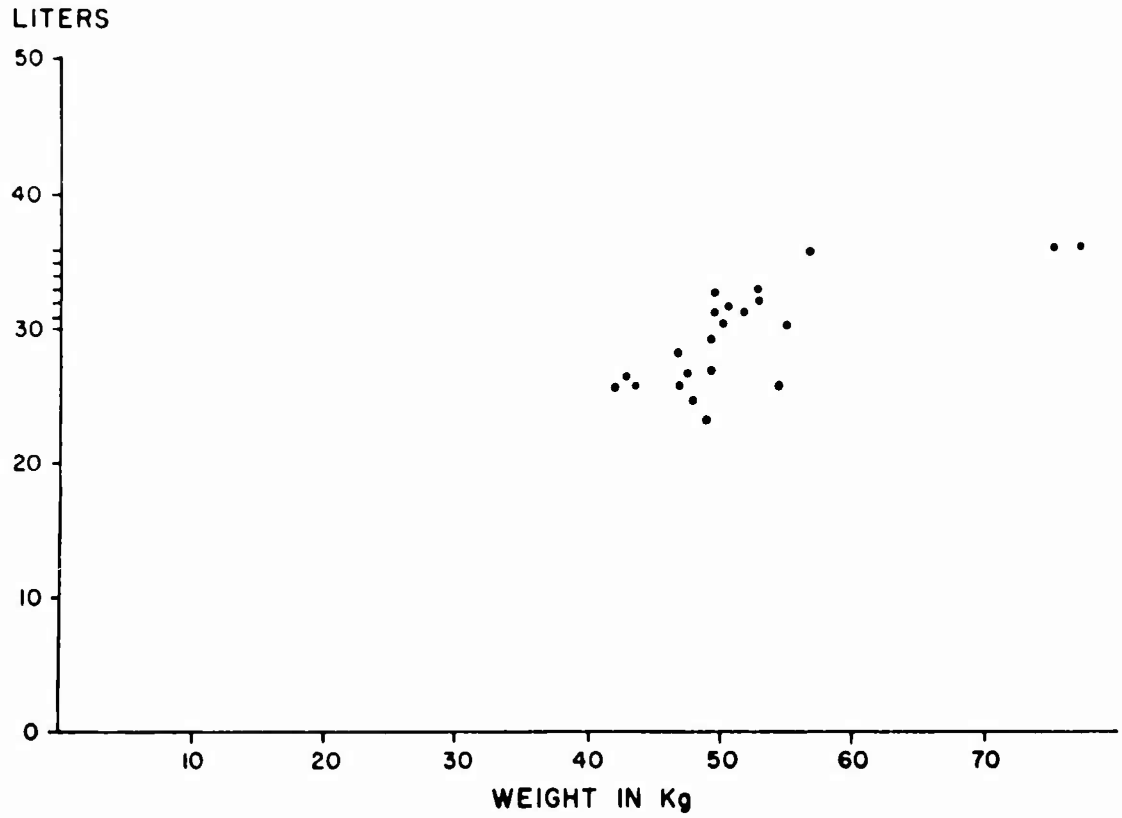
Age group	Male	Female
13.30 years	36	45
31.60 years	44	24
51.90 years	28	16
Total	108	85

Table 2

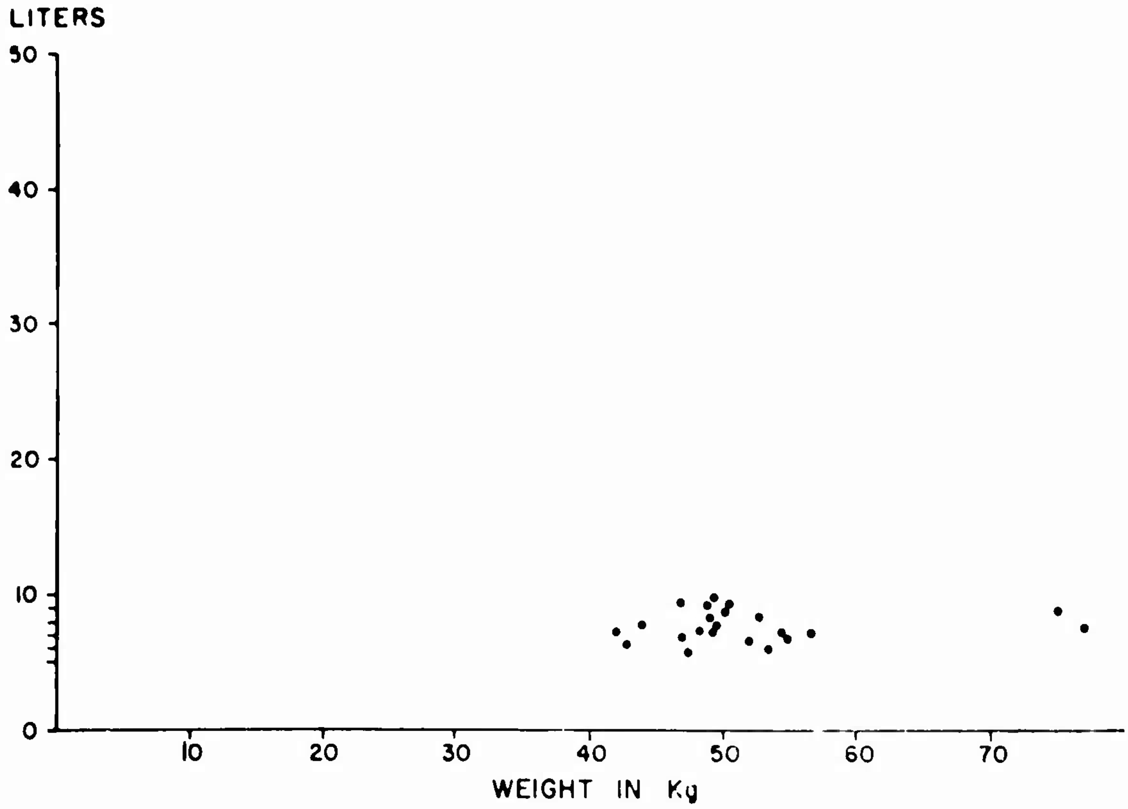
Fluid Compartments in 22 young adult Thai Subjects Confidence Limits

		Mean	SD	SE	90 Lower	90 Upper	98 Lower	98 Upper
Age	Years	26.4	11.7	2.5	22.1	30.6	20.1	32.6
Weight	Kg	52.0	8.7	1.9	48.8	55.2	47.3	56.6
Total body water	Liters	29.068	4.323	0.921	27.4826	30.653	26.745	31.391
	% B.W.	57.3	5.9	1.3	55.1	59.4	54.1	60.4
Extracellular water	Liters	7.703	1.151	0.245	7.281	8.125	7.085	8.322
	% B.W.	15.1	2.9	0.6	14.1	16.2	13.6	16.9
Intracellular water	Liters	21.365	3.808	0.812	19.963	22.761	19 319	23.410
	% B.W.	41.6	6.8	1.4	39.1	44.1	37.9	45.2
Plasma volume	ml	2242	316	67	2126	2358	2072	2412
	ml/kg	43.4	4.6	1.0	41.7	45.1	40.9	45.9
Red cell mass	ml	1431	199	42.5	1359	1505	1324	1538
	ml/kg	27.8	3.2	0.7	26.6	29.0	26.1	29.5
Total blood volume	ml	3674	488	104	3495	3853	3411	3936
	ml/kg	71.3	7.2	1.5	68.6	73.9	67.4	75.1
Hematocrits	peripheral	42.9	7.1	1.5	40.3	45.5	39.0	46.7
	TotalBody	39.0	2.3	0.5	38.1	39.8	37.8	40.2

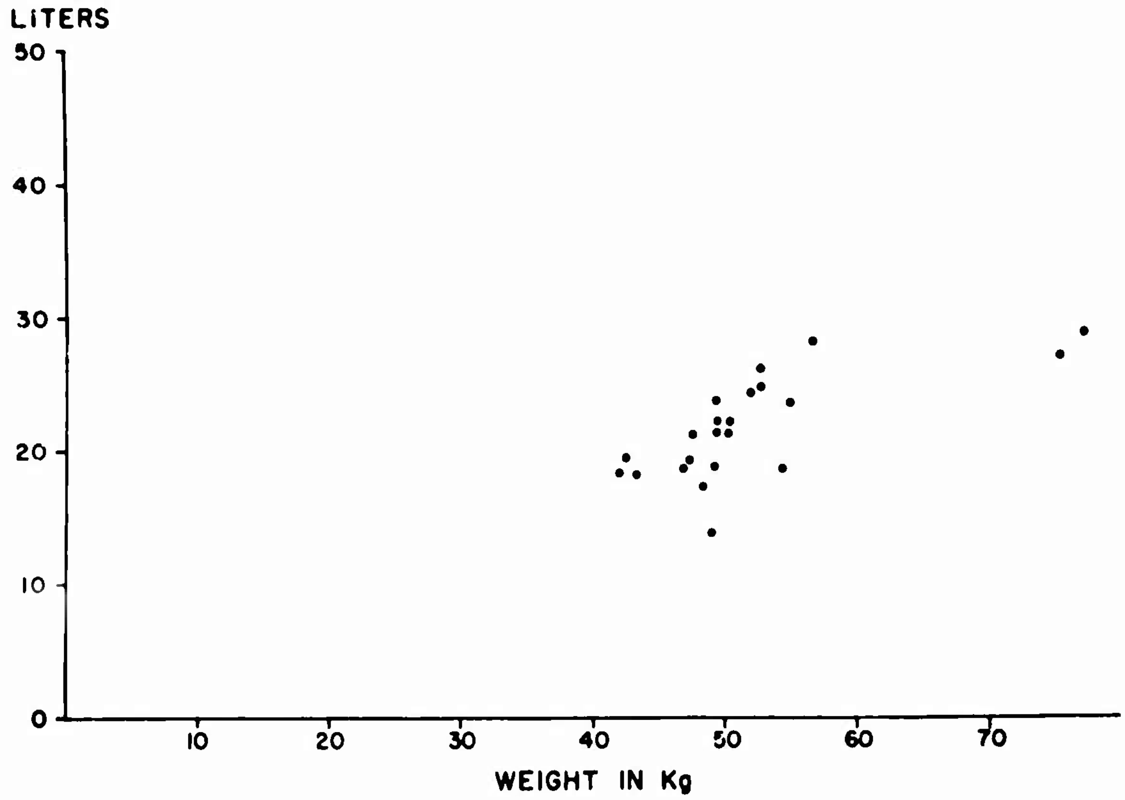
TOTAL BODY WATER MALES 13-30 YEARS IN AGE.



EXTRA CELLULAR FLUID MALES 13-30 YEARS IN AGE.



INTRA CELLULAR FLUID MALES 13-30 YEARS IN AGE.



2. Title: Evaluation of Renal Function in Normal Thai Children

Principal Investigator: Channivat Kashemsant, M.D.

Associate Investigator: Sangchan Satrarasook, M.D.

Period of Report: 1 April 1966-March 31, 1967

Objectives: To define normal ranges of renal function in Thai children for anticipated studies of a variety of disease processes.

The study includes:

1. Diurnal variation and urinary excretion pattern of electrolytes, nitrogenous substance, calcium and phosphorus.

2. Concentration and dilution abilities

3. Glomerular filtration rate (Inulin, creatinine clearance) and renal plasma flow (PAH clearance)

4. Sweat composition

5. Plasma cholesterol, protein, osmolality, electrolytes, uric acid, Urea Nitrogen, creatinine calcium and phosphorus.

Description: Thirty six healthy children, 17 females and 19 males aged 6-12 years were studied at CRC. All subjects were put on normal diet and activity. Three consecutive days of two-period urine (7AM-7PM, 7PM-7AM) were collected and sent for urinary metabolites. Blood were collected each day at 1 PM, and 1 AM and sent for chemistry.

Inulin and PAH clearances were performed in an ordinary fashion with simultaneous creatinine clearances.

Concentration test was performed by using maximal concentration ability after 22 hours of water deprivation and injection of aqueous pitressin. Dilution ability was obtained after ingestion of water and alcohol.

Sweat test was obtained after application of heat.

Progress: Analysis of data revealed the following:

1. Total daily urinary excretion:

	No Subjects	Actual ± S.D.	per 1.73M ± S.D.	Normal Adult* (Text)	Normal Thai Adult Sitprija et.al.**
Volume (ml)	35	1290 ± 424	2533 ± 812	700-3000	914 ± 6
Osmolality (Osm/L)	35	293 ± 96	—	600	517 ± 5.7
Osm./day	35	360 ± 101	722 ± 149	1200	446.7 ± 115.5
Na (mEq)	35	81.3 ± 49.8	167 ± 56	100-200	79.9 ± 7
K (mEq)	35	14.5 ± 5.01	30.6 ± 13.2	30-50	18.2 ± 3.9
Cl (mEq)	35	86.3 ± 28.2	177 ± 55	100-250	88.1 ± 21.3
Uric acid (mg)	33	342 ± 100	687 ± 164	290-750	—
Creatinine (mg)	35	399 ± 156	777 ± 157	300-1500	449 ± 90
Urea N. (Gm)	33	3.51 ± 1.14	6.99 ± 1.66	6-18	6.5 ± 0.9
Calcium (mg)	23	64.6 ± 31.0	140.3 ± 72.5	100-300	—
Phosphorus	23	243 ± 74.7	504 ± 118	700-1600	—
Creatinine clearance	35	—	90.8 ± 18.0	≰120 ml/min	61.2 ± 13

* From Hoffmann, White and King

** Sitprija et al, J.M.A.T. 48, 413, July 1965, (figures were interpreted as 95% confidence)

2. Concentration and Dilution Abilities

Thirty six subjects exhibited maximal concentrating abilty of 1195 ± 218 mOsm/L. It was quite interesting that urine osmolality always dropped after injection of pitressin. Minimal ditution to 49 ± 9.96 mOsm/L was observed.

3. Glomarular filtration rate and rensmal plasma flow

	No subjects	Mean ± S.D. ml/min/1.73 M^2	Normal ml/min/1.73 M^2
			male 131 ± 21.5
Inulin clearance	25	109 ± 20.9	female 117 ± 15.6
Creatinine clearance	25	105.5 ± 22.8	
			male 697 ± 1[illegible]6
PAH Clearance	24	446 ± 104	female 594 ± 102
Filtration fraction	24	0.24 ± 0.03	0.22 – 0.27

4. Sweat Composition

	No. sujects	mean ± S.D.	Normal (Anderson) adult
Sodium (mEg/L)	36	32.2 ± 12.16	27-87 (Av.65)
Potassium (mEg/L)	36	10.4 ± 4.8	10-15
Chloride (mEg/L)	34	25.1 ± 10.45	19-82 (Av.52)

5. Plasma Chemistry

	No. Subjects	Mean ± S.D.	Normal (Text)
Cholesterol mg%	37	168 ± 26.2	100-240
Total Protein Gm%	34	7.17 ± 0.31	6.3-8.0
Albumin Gm%	34	4.16 ± 0.59	4.23
Globulin Gm%	34	3.01 ± 0.72	3.34
Alpha-1 Gm%	34	0.22 ± 0.18	0.47
Alpha-2 Gm%	34	0.65 ± 0.20	0.75
Beta Gm%	34	0.74 ± 0.17	0.91
Gamma Gm%	34	1.36 ± 0.38	0.76
Osmolality mOsm/L	38	284 ± 4	270-310
Sodium mEg/L	38	139 ± 2	134-145
Potassium mEg/L	38	4.25 ± 0.51	3.5-5.3
Chloride mEg/L	38	104 ± 1	97-108
Uric acid mg%	37	3.89 0.79	male 3-6.3 female 1.7-5.5
Creatinine mg%	38	0.61 ± 0.35	0.4-1.4
Urea Nitrogen mg%	38	11.0 ± 1.82	10-20
Calcium mg%	27	9.42 ± 0.99	8.5-10.5
Phosphorus mg%	27	5.73 ± 0.89	4.0-5.5

Interpretation: 1. Thai children admitted for this study excreted relatively high urine volume and low osmolar concentration per litre. This was related to high water intake (average 2742 ml./day as water) because of hot weather. These children also excreted lower solute excretion when compared with average Americans. This can be explained by low protein diet in Thai. Low protein diet was also demonstrated by low urea and low phosphate exretion. Urinary excretion of other metabolites was within normal limits.

2. Thai subjects showed normal dilution ability and slightly lower concentration ability. Impairment of maximal urinary concentration ability has also been observed in healthy subjects who were permanent inhabitants of hot area of Israel.

3. Glomerular filtration rate and renal plasma flow were in lower limits of normal. Creatinine clearance when periodically performed by catheterization was closely similar to inulin clearances whereas 24 hrs creatinine clearance was slightly lower.

4. Sweat composition were in normal ranges.

5. Plasma protein was in normal ranges, Alpha-1 globulin was significantly lower and gamma globulin was definitely higher than standard. The high incidence of infection plays important role in high gamma globulin in this country. Others plasma chemistries were in normal limits.

SEATO MEDICAL RESEARCH STUDY ON RESPIRATORY VIRUSES

Coordinator: Philip K. Russell, LTC MC

Principal Investigators: Ch[illegible]inthorn Suvongse, M.D.
Rapin Snitbhan, M.D.

Associate Investigators: Chaiyan K. Sanyakorn, M.D.
Dumrong Chiewsilp, M.D.

Period of Report: 1 April 1966-31 March 1967

General Information:

The major effort in the respiratory virus field during the period of this report was concerned with development of techniques, human cell lines, and reference antisera for isolation and identification of respiratory viruses.

Human embryonic kidney and human embryonic lung cells at low passage level (2nd to 6th) are now being regularly produced in sufficient quantities for routine use. A new Hela cell line suitable for propagation of rhinoviruses was obtained. Typing sera for several respiratory viruses including rhinoviruses is now available for use.

A preliminary program for determining the etiology of viral respiratory illnesses and gathering basic epidemiologic information on transmission of respiratory viruses was begun in February 1967. Patients studied were children under 12 years of age coming to the MCH Clinic, Din Dang with upper respiratory infections. Clinical history throat and rectal swabs for virus isolation, and acute and convalescent sera were obtained on five patients each week. Virus isolation was attempted using four types of cell culture human embroyonic kidney, human embryonic lung, Hela, and BS-C-1. Cell cultures were observed for CPE and tested for hemadsorption. At the time of this report isolation attempts have been completed on 34 patients. CPE producing viruses have been recovered from 12 of the 34 patients but have not yet been identified.

It is planned to continue this program for at least one year to obtain preliminary observations on seasonal patterns of respiratory virus transmission in Bangkok.

SEATO MEDICAL RESEARCH STUDY ON Rickettsial Diseases in Thailand.

Coordinators:	Samrit Jatinandana, Captain, MC, RTN* Sittiboon Puranavej, Wing Commander, MC, RTAF** Dan C. Cavanaugh, Lieutenant Colonel, MSC USA
Principal Investigators:	Vichai Sankasuwan, Major, MC, RTA*** Pichitra Pongpradit, MD Premthavi Bodhidatta, Lieutenant, MC, RTAF****
Assistant Investigators:	Bospan Prakoppanichakij, B sc. (Pharm) Suparasri Pumsuwan, B sc. (Pharm) Bennett L. Elisberg, MD*****
Period of Report	1 April 1966-31 March 1967

Objective:

To determine the distribution and seasonal variation of Rickettsial diseases in Thailand; identify arthropod vectors and mammal reservoirs and alternate hosts, and serve as required as consultative laboratory.

Description:

A search for evidence of infection of man and other animals by rickettsiae of scrub typhus, murine typhus, Q-fever and the spotted fever group is being carried out in Thailand by means of isolation attempts and serologic methods.

Small mammals (usually rodents) are trapped in selected areas. Ectoparasites are collected, identfied and pooled. Tissue specimens and ectoparasite pools are inoculated into white mice or guinea pigs for isolation attempts. Domestic animals, and in some cases, human residents, are bled for serologic study.

In a few instances blood from hospitalized human cases of fever of unknown origin (F.U.O.) were inoculated into white mice for attempted isolation.

Progress:

1. Scrub Typhus. In order to complete the seasonal study of scrub typhus in animals from selected area, trips to Arranyaprathet, Nan and Chieng Rai were conducted during summer, winter and rainy season.

* Deputy Director, Thai Component, SEATO Medical Research Laboratory
** Chief Division of Research (Thai Component)
*** Chief Investigator (Thai Component)
**** Division of Research (Thai Component)
***** Chief Rickettsial Disease Department, WRAIR

Leptothrombidium (L) *deliense* chigger population is highest during the rainy season which corresponds to the prevalence of scrub typhus infection in trapped animals. At Nan, Loei and Chieng Rai where *Leptothrombidium* (L) *scutellaris* are present, an attempt to isolate scrub typhus rickettsiae from this chigger and the small mammals trapped in the same areas were unsuccessful (Table I). This chigger is known to be a main vector in Japan.

Attempts to recover *R. tsutsugamushi* from patients with F.U.O. were made at Bangkok Army Hospital, Overbrook Hospital, Chieng Rai and Korat Provincial Hospital. Fifteen strains of *R. tsutsugamushi* were isolated from 149 specimens of blood (Table II). It should be noted that at Korat, where a scrub typhus outbreak occured in 1965, 11 strains of *R. tsutsugamushi* were isolated from 91 civilian patients with F.U.O. during November-December 1966. No skin rashes were observed and only 3 of 15 patients exhibited eschar. It is likely that the majority of scrub typhus cases escape physicians' recognition.

2. Murine Typhus. Commensal rats and their fleas were collected during the rickettsia survey trips, and processed to recover *R. mooseri.* Twety-nine strains tentatively identifjed as *R. mooseri* were isolated from 1,077 rats trapped in 10 provinces (Table I), indicating a wide distribution of this rickettsia in Thailand.

Definitive isolation of *R. mooseri* was made from a patient diagnosed as having bronchitis at Chieng Rai and presumptive isolation was also made from another patient diagnosed as enteric fever at Korat. Rash, supposedly the most characteristic sign, was not observed in these 2 patients.

3. Q-fever During a scrub typhus survey, blood was taken from trapped animals in various provinces for Q-fever serology. One thousand, one hundred and thirty three animals, mostly rodents, were examined by mean of CF test for Q-fever using Nine Mile strain Q-fever antigen (Lederle) with 6.1% positive. (Table IV).

Attention should be drawn to the Bandicoot rats (*Bandicota indica*). From 32 rats of this species collected in Lopburi, 8 had CF antibodies to Q-fever, and six strains of *Coxiella burnetii* were isolated. This is the only kind of rat from which Q-fever rickettsiae have been isolated in Thailand.

Serological survey of large domestic animals was carried out. Four thousand three hundred and ninety four cattle, 259 sheep, 730 goat and 1105 dog sera were examined for the evidence of Q-fever infection (Table V). The high rate of seropositives found among dogs in Bangkok and Samutsakorn awaits confirmation. It is possible this unusual finding may be spurious. Studies relative to the probability of transmission of Q-fever to man are being conducted.

4. Spotted Fever. An attempt to recover spotted fever group rickettsia from 684 rodents tissue and 2099 ticks collected in 10 provinces was unsuccessful.

TABLE I

ISOLATION OF R. TSUTSGAMUSHI FROM SMALL MAMMALS TRAPPED AT NAN, LOEI AND CHIENG RAI

Locality	Specimen	Number Animals	Pools Tested	Pools Positive	Percentage
Chieng Rai	Rat Chigger	193 200	94 4	0 0	0 0
Loei	Rat Chigger	189	82	1	0
Nan	Rat Chigger	56 1075	29 7	2 0	4 0

TABLE II

ISOLATION OF R. TSUTSUGAMUSHI FROM PATIENTS WITH F.U.O.

Locality	No. Specimen of Blood	No. Isolations
Chieng Rai Hospital	53	3 (5.7%)
Bangkok Army Hospital	6	1 (16%)
Korat Hospital	90	11 (12%)
Total	149	15 (10%)

TABLE III

TENTATIVE ISOLATION OF RICKETTSIA MOOSERI FROM COMMENSAL RATS (RATTUS RATTUS, R. EXULANS AND RAT FLEAS (XENOPSYLLA CHEOPIS IN VARIOUS LOCALES IN THAILAND.

Locality	Specimen Tested	Total No. Tested	No. of Pools Tested	No. of Pools Positive for R. mooseri	Source of the Isolate
Chieng Rai	Rats	115	34	0	
	Fleas	164	9	1	*Xenopsylla cheopis*
Ubol	Rats	95	23	4	4-*R. exulans*
	Fleas	98	4	0	
Nan	Rats	17	4	2	2-*R. exulans*
	Fleas	28	6	0	
Loei	Rats	92	25	4	1. *R. rattus* 3. *R. exulans*
	Fleas	503	11	1	*X. cheopis*
Samutsakorn	Rats	69	16	1	*R. exulans*
	Fleas	26	7	0	
Korat	Rats	285	30	5	*R. exulans*
Nongkai	Rats	91	16	5	*R. exulans*
Udorn	Rats	145	21	4	*R. exulans*
Tak	Rats	86	17	3	*R. exulans*
Chiengmai	Rats	82	18	1	*R. exulans*
Total Rats		1,077	204	29	
Total Fleas		819	37	2	

TABLE IV

Q—fever Complement Fixing Antibody* in Sera Collected from Small Mammals in 11 Provinces of Thailand.

Mammal	Chieng-Rai	Nan	Loei	Udorn	Nakorn panom	Ubol	Lopburi	Arranya pathet	Yala	Pattani	Narathi was	Totals
T. glis		0/19**	5/26			0/23	0/5	3/8	0/3	1/1	0/6	9/91
R. rattus	2/72	0/4	1/38	21/286	6/12	0/19	4/38	0/18	1/26	0/13	2/32	37/558
R. exulans	2/44	0/7		0/11	8/65	0/11	0/4	0/10	0/5	0/2	0/5	10/164
R. rajah		1/16	1/19	0/1		0/27		0/2	0/1	0/8	1/39	[illegible]/113
R. berdmorei	0/1	0/2	0/8	0/17				0/3				0/31
R. niviventer			0/4							0/1		0/5
R. sabanus											0/1	0/1
R. cremoriventer						0/3			0/1	1/2		1/6
R. norvegicus								0/52				0/52
B. indica	0/3	0/1	0/6	0/3			8/36	1/9				9/58
S. murinus								8/28				8/28
M. berdmorei	0/1	0/4		0/1			0/1	0/1				0/8
A. javanica	1/3		0/1	0/2								1/6
M. personata			0/2									0/2
C. notatus											0/1	0/1
C. caniceps									0/1	0/2	0/2	0/5
Monitor											0/2	0/2
R. mulleri											0/1	0/1
Bird											0/1	0/1
TOTAL	5/124	1/53	7/104	21/321	14/77	0/83	12/8[illegible]	12/131	1/37	2/29	3/90	78/1133

* sera reactive at a 1/5 dilution.
** Number positive/number tested.

TABLE V

PREVALENCE OF Q FEVER COMPLEMENT FIXING ANTIBODY IN DOMESTIC ANIMALS, BY SPECIES AND LOCALITY.

Species	No. Tested	Positive	Percentage
Cattle	4394	266	6.05
Sheep	259	8	3.09
Goat	730	17	2.33
Dog	1105	310	28.05
Total	6488	591	39.52

DISTRIBUTION OF Q FEVER IN DOMESTIC ANIMAL IN THAILAND

Province	Cattle	Sheep	Goat	Dog	Horse
Nan	1/124	—	—	—	—
Lopburi	3/122	—	—	—	—
Samutsakorn	—	—	—	11/33	—
Cholburi	—	—	—	—	0/114
Bangkok	241/2609	3/59	3/48	299/1072	—
Nakornpathom	—	2/72	2/135	—	—
Rajburi	1/20	2/40	0/40	—	—
Petchburi	—	2/33	2/33	—	—
Lampang	5/137	—	—	—	—
Uthaithani	1/39	—	—	—	—
Lampoon	0/135	—	—	—	—
Sukothai	0/68	—	—	—	—
Pitsanulok	0/130	—	—	—	—
Nakornsawan	0/179	—	—	—	—
Karnchanaburi	—	0/55	0/113	—	—
Petchaboon	0/24	—	—	—	—
Pichit	0/33	—	—	—	—
Singburi	1/129	—	—	—	—
Prae	1/409	—	—	—	—
Korat	1/24	—	—	—	—
Srisaket	0/19	—	—	—	—
Chainart	1/132	—	—	—	—
Maurklek	0/61	—	—	—	—
Total	266/4394 6.05%	8/259 3.09%	17/730 2.33%	310/1105 28.05%	0/114

SEATO MEDICAL RESEARCH STUDY ON TREMATODES

Coordinator: Robert S. Desowitz, Ph.D.

Principal Investigators: Robert S. Desowitz, Ph.D.
Chamlong Harinasuta*

Associate Investigators. Mongkol Krustrachue**
Charin Chesdaphan
Suchart Jetanesen***

Objective:

Cases of schistosomiasis due to S. japonicum have recently been discovered in the Mekong River area of Laos bordering N.E. Thailand (Most, 1966). Since any new focus of the disease in S.E. Asia is a cause for concern, an investigation on the epidemiology of the infection in N.E. Thailand was undertaken. This report gives the results of the initial survey.

Description and Results.

Patients in the Ubol Provincial Hospital and villagers living between Ubol and the Thai-Laotian border were studied. All individuals were skin tested with an adults S. mansoni antigen according to the method of Kagan, et al (1961). An attempt was made to obtain MIF preserved stool specimens from all individuals skin tested. The stools of those individuals who showed a positive skin test, i.e., a wheal at least twice the size of the saline controls, were examined by formalin-ether concentration and sedimentation followed by the hatching of any miracidia that might be present. The results are shown in the following table.

	Number examined	Number skin test positive	Number parasitologically positive
Thai patients in Ubol hospital	43	3 (7.0%)	0
Laotian patients in Ubol hospital	6	4	1
Chongmek village	73	7 (9.6%)	1
Fangloom village	53	1 (1.9%)	0
Phiboonmangsaharn village	98	8 (8.2%)	0
Total	273	23 (8.4%)	2 (0.7%)

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** School of Tropical Medicine, University of Medical Sciences, Bangkok, Thailand
*** Ministry of Public Health, Bangkok, Thailand

The four skin testive Laotian patients in the Ubol Hospital were suffering from splenomegaly of undetermined cause. In one of these patients, a 33-year old female, miracidia were found on hatching, and on many occasions S. japonicum eggs were seen in the concentrated stools. In the second parasitologically positive case, a 28-year old Thai female from Chongmek, several miracidia were seen by the hatching technique, and S. japonicum ova were found after repeated examination of the concentrated stool specimens.

It would thus appear from the results of this preliminary study that, while infections are present in this area of Thailand, they are very light, and that the hatching technique would seem the best method for their detection. Interpretation of the skin test reactions is difficult in view of the possible cross-reaction in people sensitized to bovine and other animal schistosomes. It is also probable that man is, at present, only incidentally infected and that the main cycle of infection is maintained by one or more animals in the area. However, there is a vast development program now being implemented in N.E. Thailand with construction of many dams for agricultural irrigation. This might well change the enviromental conditions to favor an increase of human infection. In view of this, it would seem important to determine not only the true extent of the epidemicity in man, but also the vector and possible animal reservoirs.

Publication: Desowitz, R.S., Harinasuta, C., Krustrachue, M., Chesdaphan, C. and Jetanesen, S. (1967) Trans. Roy. Soc. trop. Med. & Hyg., 61, 153. Desowitz, R.S., Harinasuta, C., Krustrachue, M., Chesdaphan, C., and Jetanasen, S., 1967. The results of a stool and skin test survey for schistosomiasis in villages near the Mekong River of North-East Thailand. Trans. Roy. Soc. Trop. Med. Hyg., 61, 153-154.

SEATO MEDICAL RESEARCH STUDY ON VENEREAL DISEASES

Coordinator:	Robert L. Taylor, LTC, MSC, Chief, Department of Bacteriology and Mycology
Principal Investigators:	Kenneth Gould, LTC, MC, USAF* Robert L. Taylor, LTC, MSC
Assistant Investigators:	John C. Bell, SSG, USA Mrs. Panyasri Benjadol, M.S. Mrs. Kanchana Boonchai, B.S.
Period of Report:	1 April 1966 March 1967

* Director, Professional Services, 355th Tactical Dispensary, Takhli

Study Report

Title: Studies on the causes of Penicillin-failures in the treatment of gonorrhea

Principal Investigators:
Kenneth Gould, LTC, MC, USAF
Robert L. Taylor, LTC, MSC

Assistant Investigators:
John C. Bell, SSG, USA
Mrs. Panyasri Benjadol, M.S.
Mrs. Kanchana Boonchai, B.S.

<u>Objective</u> The objectives of this study were to investigate reported penicillin-failures in the treatment of gonorrhea in terms of antibiotic sensitivity of <u>N. gonorrhoeae</u> and concomitantly occuring organisms. Laboratory support was provided the U.S.A.F. dispensary, Takhi, Thailand, in an attempt to better evaluate the extent of therapeutic problems and to perhaps determine an effective therapeutic regimen.

A limited number of antibiotic sensitivity tests were conducted on isolates of <u>N. gonorrhoeae</u> during this reporting period. All of these isolates were from caucasian males stationed at Takhli RTAFB (central plains area). The range of penicillin was 0.06 to .72 mcg/ml, with a median of .18 mcg/ml. The range of tetracycline sensitivity was 1.0 to 2.8 mcg/ml, with a median of 1.8 mcg/ml. (Table I). These data are too limited to make comparison with the antibiotic sensitivities of <u>N. gonorrhoeae</u> determined in 1965. A concerted effort will be made during the next two months to obtains from cases of gonorrhea occurring in both males and females at Korat (central plateau), Bangkok, Udorn (northeast), Ubol (northeast), Songkhla (south), and Sattahip (southeast).

A study was initiated by LTC Kenneth Gould at the USAF dispensary, Takhli RTAFB, in order to evaluate the efficacy of 7 therapeutic regimens for urethral disease The need for such a study was obvious since there is limited coordination and uniformity of policy on the treatment of gonorrhea among the military stationed in Thailand. The Department of Bacteriology & Mycology furnished the laboratory support for this study, and SSG John Bell was placed on temporary duty at Takhli to provide this support. <u>N. gonorrhoeae</u> cultures were returned to SMRL for confirmatory studies. The following regimens were studied: A-procaine penicillin, 2.4 M units IM STAT; B-procaine penicillin, 2.4 M units IM STAT X 2 days; C-procaine penicillin, 2.4 M units IM STAT plus tetracycline, 250 mgm p.o. QID X 5 days; D-tetracycline, 250 mgm p.o. QID X 5 days; E-tetracycline, 2.0 gms p.o. STAT (single dose); F-procaine penicillin, 1.2 M units IM STAT plus tetracycline, 250 mgm p.o. QID X 5 days; and G-tetracycline, 500 mgm p.o. QID X 5 days. A brief summary of this study will be included since this laboratory acted only in a collaborative capacity. A total of 730 patients compaining of urethritis were seen in the dispensary, however, only 288 of these met the requirements of the protocol and were placed on one of the above therapy regimens. The most effective regimens were C, G, B, and F, with essentially identical cure rates (83.3% 88.7%). Regimens A and E were discontinued after preliminary results showed them to be inadequate. These data will be collated and submitted for publication by LTC Kenneth Gould.

Table II lists the organisms isolated from 208 males with urethritis. <u>N. gonorrhoeae</u> was isolated from 73.6% which is an unusually high recovery. Perhaps the reason for this success, was the great care exercised in the processing of these cultures. Specimens were inoculated and incubated immediately after collection. The organism most frequently associated with <u>N. gonorrhoeae,</u> or in combination with other

organisms, was Staphylococcus epidermidis (144/208, 69.2%). Mimae-Herella, which have been implicated as etiologic agents in penicillin resistant urethritis, were isolated from only 9 patients. Isolation of N. gonorrhoeae was also made from two of these 9 patients. The significance of staphylococci in urethritis requires further investigation in terms of its ability to perpetuate a urethritis after the initiating N. gonorrhoeae have been eliminated by penicillin therapy. These data suggest that gonorrhea in South East Asia may frequently be complicated by several concomitantly occurring organisms which could influence not only the course of the disease but also the therapeutic efforts.

Early in this reporting period, an investigation was initiated to evaluate a rapid (10-15 minute) presumptive diagnostic test for gonorrhea. This test, as reported (Pub. Health Rep. 81:318, 1966) uses commercially available paper-test-strips for the detection of cytochrome oxidase in urethral exudates as a presumptive test for N. gonorrhoeae. A simple, reliable, inexpensive and rapid diagnostic aid for gonorrhea has practical application in many military dispensaries where adequate laboratory support is not available to the clinician. For these reasons, evaluation of this technique was undertaken. A collaborative study was begun with the USAF dispensary, Don Muang in August 1966. After several months, it became obvious that the difficulties involved in maintaining a fresh supply of media and transportation for the return of inoculated media resulted in an unsatisfactorily low percentage of isolations of N. gonorrhoeae. A similar study was then undertaken at the Takhli USAF dispensary. To standardize the techniques and to handle the extra workload, SSG John Bell from the Department of Bacteriology & Mycology was assigned temporary duty at Takhli.

A history was obtained from each patient, the paper-strip oxidase test performed, a gram-stained smear prepared, and cultures inoculated and incubated immediately. The results of these three tests (oxidase smear & culture) were compared to determine the reliability of the oxidase test as a rapid diagnostic aid. Urethral exudate was inoculated onto the following media; chocolate agar (GC medium plus hemoglobin, supplement B, ristocetin and polymyxin B sulfate); blood agar; and Mimae-Herella agar. Approximately 100 specimens were also inoculated onto PPLO agar. Cultures were incubated at 37°C, with 5% added CO_2, for 48 hours. Plates were examined at 24 hours for sufficient growth, and suspicious colonies of N. gonorrhoeae subcultured for confirmation at SMRL. Organisms other than N. gonorrhoeae were also identified. Occasionally, difficulties with transportation of the fastidious N. gonorrhoeae subcultures from Takhli to Bangkok resulted in the loss of viability. Therefore, more significance had to be placed on the identification procedures used at Takhli. The capability for detection of N. gonorrhoeae by fluorescent antibody microscopy was developed at SMRL. Subsequently, duplicate smears were sent to Bangkok for examination by this procedure.

A summary of data obtained from 318 caucasian males, reporting to the dispensary with urethral disease, shows an 89.30% (284/318) correlation of the oxidase test with smear or culture. There were, however, 9.43% (30/318) of the patients who had a positive oxidase test and a negative smear and culture The oxidase test results were, therefore, considered to be "false positives" among these individuals. Negative oxidase tests were obtained in only 4 persons (1.26%) with either positive smears or from whom N. gonorrhoeae was recovered. Our data show a closer agreement of the cytochrome oxidase test with smear and culture than found in the original report (89.3% vs 78%). The closer agreement in our study is undoubtedly due to the population studied, since most of the urethral discharges were classical gonorrhea. The majority of the 9.4% "false positives" were noted among patients with scanty or non-purulent discharges. The cytochrome oxidase test is certainly not the best diagnostic technique available, but might have a place in small dispensaries where adequate laboratory facilities are not available. The oxidase test in this study approachs the reliability of the gram-stained smear (89.3% vs 94.7%) even when performed by an experienced technician. The low (1.26%) "false negatives" might make this a useful screening test for ruling out N. gonorrhoeae.

A preliminary investingation was begun to determine the reasons for the "false positive" oxidase reactions. Urethral infections with organisms, other than N. gonorrhoeae, which produce cytochrome oxidase (i.e. pseudomonas, Aeromonas, vibrios, Alcaligenes and Flavobacteria) were ruled out on the basis

of the culture results. Emperical testing showed WBC's, from the buffy coat of peripheral blood, capable of producing a typical blue color on the oxidase strip within the 10 to 15 minute period of the test. Additional testing is required to determine whether intact WBC's are capable of releasing cytochrome oxidase, or whether they must be ruptured before the enzyme can be release. Pus from a variety of sources was cultured and tested for cytochrome oxidase activity. Most of the specimens, even when not containing the organisms listed above, produced a blue color, but usually the time required was from 30 minutes to 2 hours. It, therefore, becomes extremely important to standardize the time for reading the test strips, and to restrict all readings to less than 30 minutes. An interesting observation which requires confirmation and further investigation, is the finding that fluid from blisters will produce a positive cytochrome oxidase reaction within 10 to 15 minutes after application to the test paper.

Summary — Antibiotic sensitivities of N. gonorrhoeae were determined on a limited number of strains and none were found to be exceptionally resistant to either penicillin or tetracycline. Seven therapeutic regimens for urethritis were evaluated among 288 caucasian males, and five regimens were found to be of equal efficacy (83.3% – 88.7%). Two regimens were inadequate and dropped from the study. N. gonorrhoeae was the predominating organism (73.6%) recovered from 208 males with urethritis. The organism most frequently associated with N. gonorrhoeae, or in combination with other organisms was S. epidermidis (69.2%). Mimae-Herellea were isolated from only 9 patients. Isolation of N. gonorrhoeae was also made from 2 of these 9 patients. A simple, inexpensive, rapid diagnostic aid, cytochrome oxidase paper test strips, were found to have good correlation (89.3%) with gram-stained smears and cultures. Only 1.26% of the oxidase tests could be considered as "false negatives", whereas 9.4% were "false positives". The good correlation can be attributed in part to the population studied, since the percentage of classical gonorrhea was high and a minimum number of patients had scanty discharges or "NSU". It was among the latter patients that the majority of the false positives were noted. This test may have application in remote areas where laboratory support is minimal or non-existant.

Table I

Antibiotic Sensitivity of Isolates of Neisseria gonorrhoeae Isolated in Thailand

	Conc. of Drug (mcg/ml)									
	.72	.6	.48	.36	.30	.24	.18	.12	.06	.03
Penicillin	1	–	–	–	1	3	14	7	1	–

Median .18 mcg/ml

	Conc. of Drug (mcg/ml).									
	2.8	2.6	2.4	2.2	2.0	1.8	1.6	1.4	1,2	1.0
Tetracycline	1	7	2	3	1	3	2	3	3	2

Median 1. 8 mcg/ml.

Table II

Organisms recovered from 208 Males with Urethritis

Organisms	No. isolates
N. gonorrhoeae	46
N. gonorrhoeae + Staph. epidermidis	41
" " + alpha streptococci	16
" " + micrococci	12
" " + staphylococci + alpha streptococci	11
" " + diphtheroids	10
" " + staphylococci + enterocacci	4
" " + diphtheroids + alpha streptococci	3
" " + staphylococci + klebsiella	2
" " + alpha streptococci + E. coli	1
" " + pseudomonas	1
" " + mimae-herella group	2

The following organisms were isolated simgle and in various combinations

Staphylococcus epiaermidis	103
Staphylococcus aureus	6
Alpha streptococci	49
Enterococci	41
Micrococci	37
Diphtheroids	37
Pseudomonas	6
Candida	6
Klebsiella-Aerobacter	2
Hemophilus	4
E. coli	3
Mimae-Herella group	7
No growth	26

SEATO MEDICAL RESEARCH STUDY ON ZOONOSES

Coordinator:	Jack S. Stanton, Major, VC
Principal Investigators:	Dan C. Cavanaugh, LTC, MSC Richard O. Spertzel, MAJ, VC Kwanyuen Lawhaswasdi, DVM
Associate Investigators:	Moufied A. Moussa, MAJ, MSC Do-Van-Quy, Ph.D.* Pichitra Pongpradit, M.D. Donald Hunter, COL, MSC** Sittiboon Puranaveja, Wong Commander, M.D. Phillip E. Winter, MAJ, MC
Assistant Investigators:	Robert W. Dewey, MSG John J. Howard, PFC
Period of Report:	1 April 1966 – 31 March 1967

* Institute Pasteur, Saigon, RVN
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2. Title: Survey for Antibodies to Hevpesviras Simiae in Man and Other Higher Primates.

Principal Investigators: Richard O. Spertzel, Major, VC
Kwanyuen Lawhaswasdi, D.V.M.

Objective: The object of this study is to determine by the use of cell culture techniques if there are people or other primates in and among SEATO animal colonies that have circulating antibodies against monkey B virus. This work was completed during the period 1 July -- 30 Sept 1966.

Summary: Primary weanling rabbit kidney cells were used to replicate an attenuated strain of monkey B virus furnished by WRAIR. Dilutions of sera drawn from monkeys, gibbons and workers in and around the animal colonies were screened for their ability to protect tube cultured monolayers from the cytopathic effect (CPE) caused by this virus. One hundred sixty-one sera were checked for the presence of this antibody. Twenty-four of these were found to be positive. The total comprised one hundred thirty-five primate sera and twenty six human sera. Ten monkey sera, eight gibbon and six human sera were found to be positive. Results of the initial findings were confirmed by a single repetition of the experiments. Experiments were performed in an attempt to assure that these were not cross reactive antibodies to herpes simplex virus. A strain of herpes simplex was obtained from the virus department. These positive sera did not neutralize this strain of herpes simplex.

Title: Plague Study in Southeast Asia

Principal Investigator: Dan C. Cavanaugh, LTC, MSC

Associate Investigators: Sittiboon Puranaveja, Wing Commander, MC, RTAF
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Do-Van-Quy, Ph.D.
Donald Hunter, COL, MSC

Objective: To perform an ecological and epidemiological plague survey in South East Asia.

Progress: A. Thailand. Plague surveys including collection of rodents and ectoparasites were carried out in the following areas of Thailand: Arranyaprathet, Bangkok, Chiengmai, Kanchanaburi, Korat, Mukdaharn, Nongkhai, Pakchong, Rajburi, Tak, Trad, Ubon and Udon. In addition rodent tissues and fleas collected by the Department of Medical Zoology from other areas of Thailand were submitted for examination; rodent tissues and ectoparasites, as well as human and domestic animal sera collected by the rickettsial project, Thai Component were similarly examined for evidence of *Pasteurella* pestis infection. Wild-caught or colonized fleas were tested for insecticide resistance.

1. Isolation of Pasteurella Pestis. Attempts to isolate *P. pestis* from rodent tissue or fleas of Thai origin have been carried out at the Institute Pasteur, Saigon, RVN. Of 90 such attempts, none has been successful. Further tissue or fleas collected during the past year remain to be processed.

2. Serologic Evidence of P. pestis Infection. Six hundred twenty-five rodent sera and 1053 human sera have been examined for the presence of hemagglutinating antibody to P. pesis. None were positive. A single positive serum, with a titer of 1:64 was found among 472 canine sera similarly tested. This positive serum came from a dog trapped by the Bangkok municipality in its anti-rabies program. The exact locality in Bangkok from which this dog came could not be determined.

3. Flea Index. Flea indices for the various regions of Thailand (Table 1) show some seasonal variation, with maximum indices being seen in those months during which human plague cases were formerly recorded. Except for Kanchanaburi, the reported flea indices refer to *Xenopsylla cheopis* on various *Rattus* spp. In the Kanchananaburi area, the predominant rodent trapped in November 1966 and February and March 1967 was *Bandicota bengalensis*; the indices for this area and time period refer to *X. vexabilis* on this rodent.

It is noteworthy that although it is commonly held that *X. cheopis* indices in Thailand rarely exceed 2, we have found an index of 3 or higher not unusual.

4. Insecticide Resistance in Fleas. Wild-caught fleas which were tested for insecticide resistance generally showed resistance to DDT and sensitivity to other insecticibes tested (Table 2). Fleas from Ban Pong, Rajburi appear to be somewhat more sensitive to DDT than those from other areas. As noted, regression lines for mortality are fitted only with difficulty in the case of wild-caught fleas, since there is considerable mortality, probably associated with the trapping procedure and handling of fleas with attendant injury. In addition numbers available for testing from any given area are often limited, preventing replicate determinations. These problems are avoided by the establishment of flea colonies, especially from trapping

areas of low flea density. Fleas collected from rodents in Thailand thus far have appeared to be either X. cheopis or X. vexabilis. These species can be readily identified and separated under a microscope, alive.

Colonization. Fleas separated by species are transferred to large screw capped jars, in which a layer of saw dust has previously been placed. Ground dog biscuit is added, as larva food. A suckling mouse is placed in the jar to provide blood meals for the adult fleas. The mouth of the jar is then closed with muslin. This simple procedure can be carried out in the field and the fleas transported to the laboratory in the colony jars.

About 2 months is required to establish a colony large enough for a series of resistance tests. Colonized fleas appear to be superior for testing purposes to wild-caught fleas, inasmuch as insecticide dose-response is more uniform and reproducible. This is probably due to varying age and vigor of the wild-caught flea population.

Flea colonies have been established using X. cheopis from Bangkok and from Korat. Table 3 summarizes insecticide resistance data obtained from these colonies.

B. Viet Nam. Studies in Nha Trang RVN are being carried out as a joint project with the Institute Pasteur and USA Medical Research Team (WRAIR) Vietnam. Nha Trang is of interest since the current plague outbreak in RVN apparently made its first appearance in that city, in 1962. No organized rodent or flea control program has been attempted there, so the city presents an undisturbed urban rodent-flea ecology.

1. Human Plague. From 1963 through November 1966, of 1084 suspected human plague cases, for which bacteriologic diagnosis was attempted by the Institute Pasteur of MRT, 525 were confirmed. Sporadic cases are reported year-round, but the majority of cases have occurred in February, March and April each year; in 1967 a major outbreak of human cases again occurred in Nha Trang, with at least 70 confirmed cases.

2. Evidence of P. pestis Infection in Rodents and Fleas. Table 4 summarizes the Nha Trang data for 1966-67. Suncus murinus, the house shrew, was the most common rodent encountered; one isolation of P. pestis was made from this source, and 6 of 16 sera tested showed the presence of hemagglutinating antibody. Rattus norvegicus, trapped in slightly lower numbers than S. murinus was the source of at least 3 isolates of P. pestis; 56 of 206 sera thus far tested were positive for P. pestis antibody. R. exulans was trapped less than half as often as the other two species; no isolates were made but 2 of 13 sera tested were positive for HA antibody.

Flea indices varied with rodent species; R. norvegious, which had the most consistent evidence of P. pestis infection, had consistently high X. cheopis index, from 6.3 to 8.5 Three of 21 flea pools from this rodent were positive for P. pestis. The flea index on R. exulans was consistently low, from 0.9 to 2.0. One P. pestis isolation was made from 21 flea pools from this species. The flea index on S. murinus was as high or higher than that on R. norvegicus (8.7) during the "plague season" and as low (2.5) as R. exulans in October. Four flea pools of 20 tested were positive for P. pestis. Data presented are incomplete, since laboratory results of the March 1967 field trip are not yet available.

3. Insecticide Resistance. Table 5 presents resistance data for fleas collected in Nha Trang in 1966-67. A high level of DDT resistance is indicated; sensitivity to other insecticides is present.

Summary and Comment.

Plague has not been detected in Thailand since 1952. Present studies will be continued and will be expanded to include possible sylvatic foci. The finding of a significant HA titer in one dog serum indicates that an enzootic focus may exist in or around Bangkok, or the presence of a rare undetected human case.

Fleas in the areas of South East Asia for which data are available show a high level of resistance to DDT, though remaining susceptible to other insecticides. Flea collection for insecticide tests must be continued to determine the most effective insecticide for use in plague control, to avoid rapid spread of newly introduced epizootic plague. In areas and at times of low flea density, colonization of fleas will insure adequate numbers of fleas are available for testing.

The epidemiologic data necessary for the control of plague in the coastal city of Nha Trang are available. Plague is present throughout the year, with a pronounced seasonal peak in human case: in February-April of each year. Plague appears to be present in the rodents and insectivores throughout the year; with some seasonal variation in R. exulans and S. murinus, but relatively constant in R. norvegicus. Fleas, almost entirely X. cheopis, from these animals, are highly resistant to DDT. Flea populations are at their highest during the "plague season" but the index on R. norvegicus is high year round. Socio-economic conditions in the area are poor; rat control is probably not feasible on a large scale. Flea reduction, using an insecticide of demonstrated effectiveness, is probably the only practical method for plague control in Nha Trang.

Table 1

Rodent Flea Indices* by Trapping Area and Month of Collection, Thailand 1966 1967.

Area / Month	Bangkok	Korat	Pak chong	Kanchanaburi	Rajbiri	Arranyaprathet	Chiengmai	Tak	Udorn	Nongkhai	Ubol
May 66	1.6	—	—	—	—	—	—	—	—	—	—
Jun	—	—	—	—	—	—	—	—	—	—	—
Jul	1.5	1.1	—	—	—	—	—	—	—	—	—
Aug	1.0	1.7	2.8	—	—	0.2	—	—	—	—	—
Sep	0.6	2.1	1.1	—	—	—	—	—	—	—	1.0
Oct	—	1.4	0.8	—				—	1.2	1.4	—
Nov	0.9	1.3	1.9	0.6**	1.8	—	—	—	—	—	—
Dec	—	1.0	1.5		—	—	3.2	2.9	—	—	—
Jan 67		1.6	2.7	—	—	—	—	—	—	—	—
Feb		—		1.3**	2.7	—	—	—	—	—	—
Mar	3.1	2.8	2.8	9.5**	—	—	—	—		—	3.7

* Except as as noted, X. choepis on various Rattus Spp.

** X. vexabilis on Bandicota bengalensis.

Table 2

Insecticide Sentivity of Wild-Caught Fleas from Former Plague Foci in Thailand.

Area of Collection	LC_{50} in DDT	% Dieldrin	Benzene Hexachloride
Ban Pong (Rajbusi)	1.2	NT	NT
Pakchong	ND	0.07	0.09
Korat	ND	0.07	0.08

Note:

ND Not determinable; regression line not fitted, 20-40% mortality in each standard test concentration.

NT Not tested.

Table 3

Insecticide Sensitivity of Colonized X. cheopis from Bangkok and Korat Thailand.

Source of Colony	LC_{50} in %		Malathion
	DDT	Benzene Hexachloride	
Bangkok	> 4.0	0.3	0.02
Korat	> 4.0	0.4	0.02

Note: For DDT 23% mortality at 4.0% conc., Bangkok fleas.
40% mortality of 4.0% conc., Korat fleas.

Each figure result of 3 replicates.

Table 4

Summary of Field and Laboratory Studies by Mammal Species and Month, Nha Trang 1966 - 1967.

Mammal Species	Month of Study	Number Trapped	Plague HA Antibody*	P. pestis Isolates*		Flea** Index
				Spleen pool	Flea pool	
Rattus Novegicus	Apr 66	68	8/34	0/6	0/4	8.5
	May	159	30/106	0/21	2/13	7.1
	Oct	102	18/66	1/10	1/4	6.4
	Mar 67	149	N.C.	N.C.***	N.C.	6.3
Rattus exulans	Apr 66	22	0/2	0/4	0/4	2.0
	May	110	2/9	0/16	0/13	1.2
	Oct	31	0/2	0/4	1/4	1.7
	Mar 67	25	N.C.	N.C.	N.C.	0.9
Suncus Murinus	Apr 66	41	0/3	0/5	1/4	8.7
	May	169	5/9	1/20	2/12	7.1
	Oct	112	1/4	0/12	1/4	2.5
	Mar 67	208	N.C.	N.C.	N.C.	5.4

Notes:
* No. positive/no. tested
** > 99% *X. cheopis*
*** At least 2 rats positive, remainder not complete
N.C. Not completed.

Table 5

Insecticide Sensitivity of Wild-Caught Fleas (99% *X. cheopis*) from Nha Trang RVN 1966-1967.

Insecticide	No. of Replicate Teste	LC_{50} in %
DDT	22	2.3
Dieldrin	15	0.1
Benzene hexachloride	5	<0.125
Diazinon	2	0.003
Malathion	6	0.023

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13. ABSTRACT

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